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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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* Out of print.

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VIRUS III ENCEPHALITIS.

By THOMAS M. RIVERS, M.D., and FRED W. STEWART, M.D.

(From the Hospital and Laboratories of The Rockefeller Institute for Medical Research.)

Plates 12 to 14.

(Received for publication, July 6, 1928.)

In 1923 Rivers and Tillett (1) described reactions in rabbits which were considered to be induced by the virus of varicella. Subsequently, however, in Rivers' (2, 3) laboratory and also in Swift's (4, 5), it was found that the reactions were caused not by the virus of varicella but by an unknown virus indigenous to rabbits which had been accidently encountered in the work on chicken-pox and rheumatic fever. Inasmuch as the spontaneous disease caused by the virus has not been recognized, no name other than Virus III has as yet been given this active agent. The term is used merely for convenience and designates the third strain of the virus encountered with which most of the work happens to have been conducted.

The character of Virus III lesions and the presence of acidophilic nuclear inclusions (3) in the injured tissues led Rivers and Tillett to do cross-immunity experiments to determine what relationship, if any, Virus III bears to herpetic virus (2). None was found. Furthermore, in 1924, rabbits were inoculated intracerebrally with Virus III to determine if it was capable of producing an encephalitis (2). A temperature above 104°F., which persisted for a week and which was much more marked in the experimental than in the control animals, was the only abnormality noted. Because of the mild reaction no further study of Virus III encephalitis was made at that time.

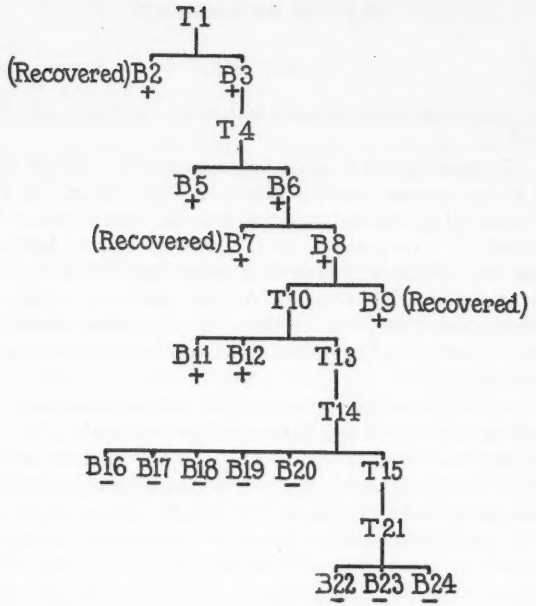
From the time of discovery of Virus III, early in 1923, until September, 1926, when emulsions of tissues containing the virus were frozen, desiccated, sealed in tubes, and stored on ice, testicular passages (approximately 300) were made at intervals of 3 or 4 days. In January, 1928, the dried virus was removed from the ice box and testicular passages were resumed. In the course of some experiments,

intracerebral inoculations with the virus were made in rabbits and signs of encephalitis, which were followed by death in a number of instances, were observed. It is with this encephalitis caused by Virus III that the present paper deals.

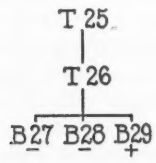
EXPERIMENTAL.

Methods and Materials.—In sealed tubes on ice, frozen and desiccated Virus III (6) retains its activity indefinitely. Its activity is also maintained for at least 6 weeks if infected testicular emulsions are mixed with equal amounts of glycerol, sealed, and stored on ice. Experiments, however, were always conducted with fresh material. Either emulsions of infected testicles or brain emulsions containing the virus served this purpose. The emulsions were prepared by grinding the infected tissues with sand in a mortar and then adding enough Locke's solution to make a 20 per cent suspension. To free the material from sand, centrifugation at low speed for 1 minute was employed. 2,000 gm. rabbits were used. 0.2 cc. of an emulsion was the amount chosen for intracerebral or for intradermal inoculation, and 1.0 cc. for intratesticular inoculation. Even though it does not so appear in the text-figures, at least two rabbits were inoculated each time the virus was passed. In working with Virus III this is necessary because an immune animal is occasionally encountered which results in the loss of the virus. The sterility of all tissues was tested by means of aerobic and anaerobic cultures. All operations were performed under ether anesthesia. Tissues for histological studies were fixed in Zenker's fluid and stained either with eosin-methylene blue or by Giemsa's method.

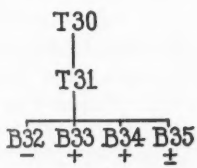
In Text-figs. 1 to 3 are outlined the methods of procedure employed in the study of Virus III encephalitis. Although the majority of intracerebral inoculations was made with testicular virus, it will be seen in Text-fig. 1 that the virus propagated itself through three successive intracerebral passages, causing in each instance definite signs of encephalitis, *e.g.*, tremor, ataxia, irritability, circling, salivation, retention of urine, generalized tonic and clonic contractions of the skeletal muscles, or paralysis. From the text-figures it will also be observed that potent testicular virus did not produce signs of encephalitis in every rabbit of certain series and that the results of intracerebral inoculations varied considerably in the different series of animals, *i.e.*, at times all the rabbits died, while on other occasions none showed signs other than a pathological increase in temperature. There is no adequate explanation for this remarkable variation in activity of the virus when inoculated intracerebrally.



TEXT-FIG. 1. Outline of procedure employed in the study of Virus III encephalitis. *T* indicates site of inoculation (testicle) and also organ emulsion (testicle) used for next passage of virus. *B* indicates site of inoculation (brain) and also organ emulsion (brain) used for next passage of virus. + indicates occurrence of definite clinical signs of encephalitis. − indicates absence of clinical signs of encephalitis other than fever. Rabbit T 1 was inoculated intratesticularly with glycerolated Virus III 5 passages removed from an animal inoculated with the desiccated material that had been stored on ice more than a year.



TEXT-FIG. 2.



TEXT-FIG. 3.

TEXT-FIG. 2. Symbols employed in manner similar to that in Text-fig. 1. Rabbit T 25 was inoculated intratesticularly with glycerolated Virus III from Rabbit T 1.

TEXT-FIG. 3. Symbols employed in manner similar to that in Text-fig. 1. Rabbit T 30 was inoculated intratesticularly with desiccated Virus III that had been stored on ice more than a year.

Rabbit B 5.

March 6. Inoculated intracerebrally with 0.2 cc. of fresh testicular Virus III from Rabbit T 4.

March 7. Temperature 104.2°; animal appears normal. March 8. Temperature 105.0°; animal appears normal. March 9. Temperature 103.4°; sick and irritable. Occasional generalized tonic and clonic contractions of the skeletal muscles. March 10. Temperature 101.0°; paralysis of hind legs; retention of urine. March 11. Paralysis; retention of urine; generalized tonic and clonic contractions of the skeletal muscles. Animal sacrificed. Brain edematous; vessels distended with blood. No evidence of a purulent meningitis. Brain fixed in Zenker's fluid. Sections stained with eosin-methylene blue and according to Giemsa's method.

Section through Hippocampal Region.—Slight general thickening of pia-arachnoid with cellular infiltration consisting of many endothelial leucocytes, a few lymphocytes, and rare polymorphonuclear cells. Occasional acidophilic nuclear inclusion (Fig. 7) in endothelial leucocytes. In places, fixed endothelial cells are prominent and rarely contain inclusions; few mitotic figures; slight perivascular infiltration of penetrating vessels. Generalized meningeal hyperemia with a moderate amount of hemorrhage. Few nuclear inclusions in cells that probably are arachnoidal fibroblasts. Hemorrhage in wall of third ventricle, accompanied by very slight reactive changes. Nuclear inclusions in cells of ependyma (Fig. 6) and choroid plexus. In foci in hippocampus are numerous typical nuclear inclusions; the cellular degeneration is associated with very little, if any, reaction.

Section through Cerebellum.—Meningeal lesions similar to those described above; marked cortical hemorrhage. Nuclear inclusions in outer cells of molecular layer. Pycnotic and fragmentary degeneration of nerve cells of granular layer (Fig. 5). Marked hyaline necrosis of Purkinje cells with pycnosis and chromatolysis of nuclei (Fig. 1). No inclusions found in Purkinje cells.

Rabbit B 6.

March 6. Inoculation similar to that of Rabbit B 5. March 7. Temperature 104°; animal appears normal. March 8. Temperature 105°; animal appears normal. March 9. Temperature 102°; sick and irritable. March 10. Paralysis of hind legs. March 11. Paralysis; generalized tonic and clonic contractions of the skeletal muscles. March 12. Paralysis; generalized tonic and clonic contractions of the skeletal muscles. Animal sacrificed. Brain edematous. Aerobic and anaerobic cultures showed no growth. Part of brain used for passage of virus, remainder fixed in Zenker's fluid for histological study.

Section through Hippocampal Region.—Diffuse meningitis; some edema, hemorrhage, and fibrin. Nearly all of the cells are endothelial leucocytes; occasional typical nuclear inclusion. Nothing of importance observed in hippocampus.

Section through Cerebellum.—Meningeal reaction similar to that described above.

Some diffuse and marked focal degeneration of Purkinje cells as indicated by the striking oxyphilic reaction involving both the nucleus and the cytoplasm; chromatolysis, karyorrhexis, and a fading out of the Purkinje cells together with adjacent cells of the molecular layer. Throughout the degenerating areas the lack of a significant degree of inflammatory response is noteworthy.

Rabbit B 7.

March 12. Inoculated intracerebrally with 0.2 cc. of fresh brain Virus III from Rabbit B 6.

March 13. Temperature 102.8°; animal appears normal. March 14. Temperature 103.4°; animal appears normal. March 15. Temperature 104.0°; sick. March 16. Temperature 104.2°; sick. March 17. Temperature 104.5°; sick. March 18. Sick. March 19. Temperature 101.2°; sick; head pulled to left and rotated. March 20. Temperature 100.5°; ataxia; loss of weight; head pulled to left; circling. March 21. Temperature 100.0°; retention of urine. March 22. Animal prostrate. March 23. Began to improve and eventually recovered.

Rabbit B 8.

March 12. Inoculation similar to that of Rabbit B 7.

March 13. Temperature 102.8°; animal appears normal. March 14. Temperature 102.4°; animal appears normal. March 15. Temperature 105.8°; sick; irritable; head retracted. March 16. Temperature 104.0°; condition worse. March 17. Temperature 105.0°; condition same. Animal sacrificed for passage of the virus and for histological studies. Cultures of the brain showed no growth.

Sections through the Point of Inoculation and also through the Hippocampus.—Diffuse meningitis—the cells chiefly endothelial leucocytes; a slight generalized invasion of peripheral cortical tissue both over the surface of the brain and also beneath the ependyma. Some perivascular thickening due to endothelial cells. Numerous characteristic nuclear inclusions in endothelial leucocytes and some in arachnoidal fibroblasts, especially in those applied along large vessels. Hyaline degeneration of scattered nerve cells in periphery of cortex. About the site of inoculation, hemorrhage, spongy degeneration of the parenchyma, and a confused cellular picture are observed. All cells are swollen; nuclei are reduced to peripheral chromatin rings with central inclusions. No other structure is sufficiently preserved to enable absolute identification of specific cell type, but it appears that all structures contain inclusions—endothelial, nerve, and glia cells. Marked degeneration of hippocampal cells; swollen nuclei; numerous typical nuclear inclusions.

Rabbit B 29.

April 19. Inoculated intracerebrally with 0.2 cc. of fresh testicular Virus III from Rabbit T 26.

April 20. Temperature 104.8°; animal wild. April 21. Temperature 104.0°; animal wild. April 22. Temperature 105.4°; animal wild. April 23. Temperature 104.5°; tremor. April 24. Temperature 103.0°; marked tremor and ataxia; circling to left; head pulled to left; salivation; twitching of muscles around mouth and of fore legs; occasional generalized tonic and clonic contractions of the skeletal muscles. Animal sacrificed for histological studies. Brain perfused with Zenker's fluid.

The usual meningitis with more polymorphonuclear cells than usually seen. Isolated Purkinje cells show typical hyaline necrosis and nuclear chromatolysis. Nuclear inclusions in ependymal cells lining fourth ventricle and in neighboring glia cells.

Very severe degenerative changes in hippocampus (Fig. 4) with nuclear inclusions in almost every cell (Fig. 2) and extensive spongy degeneration in fiber layers. Slight perivascular hemorrhage. Perivascular infiltration (Fig. 8) consisting of polymorphonuclear cells, lymphocytes, and endothelial leucocytes.

Spongy degeneration and necrosis of nerve cells with typical nuclear inclusion in thalamic region. Epithelial cells of choroid plexus contain nuclear inclusions.

Meningitis with some of the cells showing nuclear inclusions extends to the cervical cord. Slight involvement of periphery of cord and of perivascular sheaths.

Rabbit B 35.

May 19. Inoculated intracerebrally with 0.2 cc. of fresh testicular Virus III from Rabbit T 31.

May 20. Temperature 102.8°; animal seems normal. May 21. Temperature 104.9°; animal seems normal. May 22. Temperature 104.0°; sick. May 23. Temperature 104.0°; worse; tremor. Tremor persisted until May 26, when the rabbit was sacrificed. The brain was perfused with Zenker's fluid. This animal showed no signs of encephalitis except fever, tremor, and a tendency to stand rigidly in one position for long periods of time.

Meninges show lymphocytic, endothelial and plasma cell infiltration, fibrin, and hemorrhage. Nuclear inclusions in endothelial leucocytes and fixed endothelium. Perivascular lymphocytic infiltration along penetrating vessels.

Hyaline necrosis with nuclear karyorrhexis of many Purkinje cells. Nuclear inclusions in small nerve cells interspersed between Purkinje cells, in glia cells, and in endothelial leucocytes. Spongy degeneration of associated fiber layer.

Extensive spongy degeneration with nuclear inclusions in nearly every cell in the hippocampus. Some inclusions in peripheral cortical glia cells and in invading endothelial leucocytes.

A few typical inclusions in glia cells in periphery of cervical cord. Slight endothelial cell infiltration.

In addition to Virus III encephalitis this brain also showed lesions of the spontaneous encephalitis described by Wright and Craighead (7).

From the results of the experiments described above it is obvious that the active agent used is capable of producing an encephalitis in rabbits. The question naturally arises, however, as to whether the virus now under investigation is the one originally encountered 5 years ago, or whether it has become contaminated by another virus, *e.g.*, vaccine virus or herpetic virus.

Relation of Present Virus III to Original Virus III.

Repeated experimental passages of the virus in animals that are occasionally spontaneously infected make it impossible to say definitely that the original strain of Virus III has not been contaminated by a new strain of the active agent. A fortunate circumstance, however, enabled us to demonstrate that the present Virus III is identical with, or at least similar to, the original Virus III. In 1925 Rivers and Pearce (8) found that the transplantable rabbit neoplasm described by Pearce and Brown (9) is infected with Virus III and that the virus persists in the tumor and is regularly passed from rabbit to rabbit with each successive transfer of the tumor. In view of these facts, in order to establish a relationship between the present Virus III and the Virus III of 1925 it was only necessary to determine whether the tumor rabbits are refractory to the active agent now being used. For this purpose Dr. Pearce supplied 6 rabbits that had shown good growths of the tumor.

6 tumor rabbits were inoculated intradermally and intracerebrally respectively with 0.2 cc. of a fresh testicular emulsion containing virus of the same generation as Rabbit B 6 (Text-fig. 1). As controls, an animal which had recovered from encephalitis caused by our virus was inoculated in a similar manner, and 2 normal stock animals received intradermal and intratesticular inoculations. The results showed that the recovered animal and the 6 tumor rabbits had no reaction in the skin and evidenced no signs of encephalitis, while the normal animals had a very marked reaction in the skin and a high fever.

From the results of the above experiment one is justified in concluding that in all probability the virus now being used is identical with the original Virus III.

Consideration of Possible Contaminants.

The next question to arise dealt with the possibility that the emulsions containing Virus III were contaminated by the virus of vaccinia, rabies, or herpes.

Vaccine Virus.—One can be quite positive that the emulsions containing Virus III do not also contain vaccine virus, inasmuch as Dr. Pearce's tumor rabbits, not immune to vaccine virus, are completely protected against the activity of our emulsions. Furthermore, no Guarnieri bodies were observed in cells injured by our active agent.

Rabic Virus.—In making repeated passages in rabbits, it is not inconceivable that one might rarely encounter the virus of rabies. This possibility is very remote. Moreover, Dr. Pearce's tumor animals are immune to our virus, even when inoculated intracerebrally. If rabic virus were a contaminant, her animals, although immune to Virus III, would die of rabies. This did not occur, nor were Negri bodies found in the brains of rabbits dying of Virus III encephalitis.

Herpetic Virus.—The clinical and pathological picture presented by our animals at times so closely resembles that caused by herpetic virus that one naturally would like to know whether our emulsions are contaminated by the virus of herpes.

5 rabbits were chosen; 1 was a normal stock animal, the other 4 had recovered, 4 to 8 weeks previously, from Virus III encephalitis. Each animal was inoculated intracerebrally with 0.2 cc. of a brain emulsion containing H. F. herpetic virus. All of the rabbits showed the usual signs of herpetic encephalitis and were dead within 7 days.

The above experiment clearly indicates that our virus is not contaminated by herpetic virus.

DISCUSSION.

Virus III is an active, filterable agent indigenous to rabbits (2, 5). It undoubtedly causes a natural infection in these animals, yet the spontaneous disease has not as yet been recognized. As previously shown (1–3), the virus under experimental conditions produces a high fever and characteristic lesions in the cornea, testicles, and skin. Furthermore, within epithelial and endothelial cells of these lesions

acidophilic nuclear inclusions, similar to those seen in varicella and herpes, occur (3).

The studies described in the present paper clearly indicate that Virus III at times is capable of inducing in rabbits an encephalitis which clinically and pathologically closely resembles that caused by herpetic virus. The most interesting fact disclosed by the present work, however, is that the ability of the virus to produce visible evidences of encephalitis seems to vary greatly from time to time (Text-figs. 1 to 3). No adequate explanation of this striking feature is now available. The question as to whether the frequent passages of the virus under experimental conditions, with an occasional freezing and desiccation or storage in glycerol, have altered its activity cannot be answered at present.

The histopathology of experimental Virus III meningoencephalitis in rabbits resembles in part that of herpetic encephalitis. The two diseases are pathologically similar in that both produce a "chronic" type of meningitis, characterized by lymphocytic, plasma cell, and endothelial cell infiltration (Fig. 7); the perivascular sheaths of penetrating vessels may be distended by similar cells (Fig. 8). In both diseases the hippocampal region is profoundly involved; nerve cells, glia cells, and endothelial leucocytes contain characteristic acidophilic nuclear inclusions (Fig. 2); nerve cells undergo hyaline degeneration and seem to disappear rapidly, leaving a spongy, reticulated zone of ground substance (Figs. 2, 4). The adjacent fiber laminæ likewise present a soft, spongy appearance and a few polymorphonuclear and endothelial leucocytes infiltrate the region.

In Virus III encephalitis the next most prominent lesion occurs in the Purkinje cell layer of the cerebellum (Figs. 1, 5). These large cells undergo hyaline necrosis accompanied by nuclear chromatolysis, pycnosis, and karyorrhexis; no inclusions have been observed in their nuclei. Inclusions, however, frequently occur in the smaller nerve cells, in glia cells, and in reacting endothelial leucocytes in the immediate vicinity. The necrosis and disappearance of Purkinje cells leave a zone of spongy degeneration between the granular and molecular layers of the cerebellum. In some animals extensive pycnotic degeneration of nerve cells in the granular layer of the cerebellum (Fig. 5) also occurs. Some of the peculiar clinical manifestations of

the disease are probably due to the cerebellar lesions, but in view of multiple foci of brain involvement one should be cautious in relating the clinical picture to lesions in different anatomical foci.

Meningeal edema and hemorrhage, small hemorrhages at the site of inoculation, and foci of hemorrhage in the deep pontine region further complicate the picture. In some rabbits superficial cortical and subependymal encephalitis was noted; here one finds a spongy degeneration, mitosis of glia and endothelial cells, and infiltration by endothelial leucocytes.

It seems that no type of cell escapes involvement. Inclusions have been seen in nerve cells, glia cells, fixed and mobile endothelial cells, arachnoidal fibroblasts, ependymal cells, and in cells of the choroid plexus. Often one cannot distinguish between a glia cell and an infiltrating endothelial leucocyte because of the loss of characteristic nuclear structure; involved nuclei are reduced to a central, often elongated, inclusion surrounded by a clear zone limited externally by a narrow ring of altered chromatin.

The presence of inclusions, the disappearance of cells and ground substance giving rise to the peculiar soft, spongy, reticular appearance, and the minimal amount of inflammatory reaction agree well with the findings in encephalitis caused by other filterable viruses.

SUMMARY.

Virus III, an active, filterable agent indigenous to rabbits, under experimental conditions produces, in addition to lesions in the cornea, skin, and testicles, an encephalitis which is at times quite similar to that induced by herpetic virus. Virus III and herpetic virus, however, are not immunologically related.

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EXPLANATION OF PLATES.

PLATE 12.

FIG. 1. Cerebellum. Hyaline necrosis of Purkinje cells. Zenker; eosin-methylene blue. × 500.

FIG. 2. Hippocampus. Cellular degeneration; abundant inclusion bodies in nuclei. Zenker; eosin-methylene blue. × 1000.

FIG. 3. Site of inoculation. Large granular nuclear inclusion in nerve cell. Zenker; eosin-methylene blue. × 1500.

PLATE 13.

FIG. 4. Necrosis of hippocampal cells; spongy degeneration of fiber layers; hyperemia and slight cellular reaction. Zenker; eosin-methylene blue. × 115.

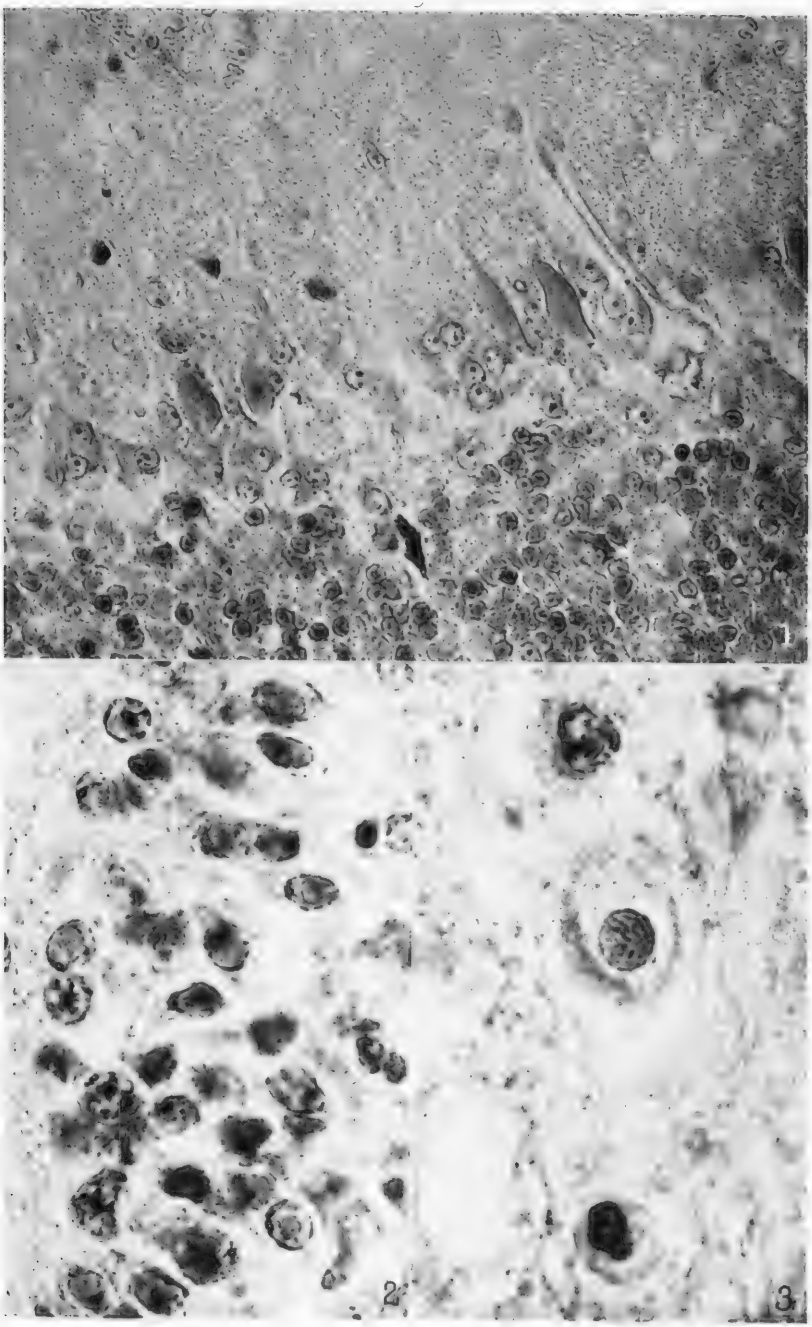
FIG. 5. Pycnotic degeneration of nerve cells of granular layer of cerebellum. Zenker; eosin-methylene blue. × 210.

PLATE 14.

FIG. 6. Inclusions in ependymal cells lining third ventricle. Zenker; eosin-methylene blue. × 1000.

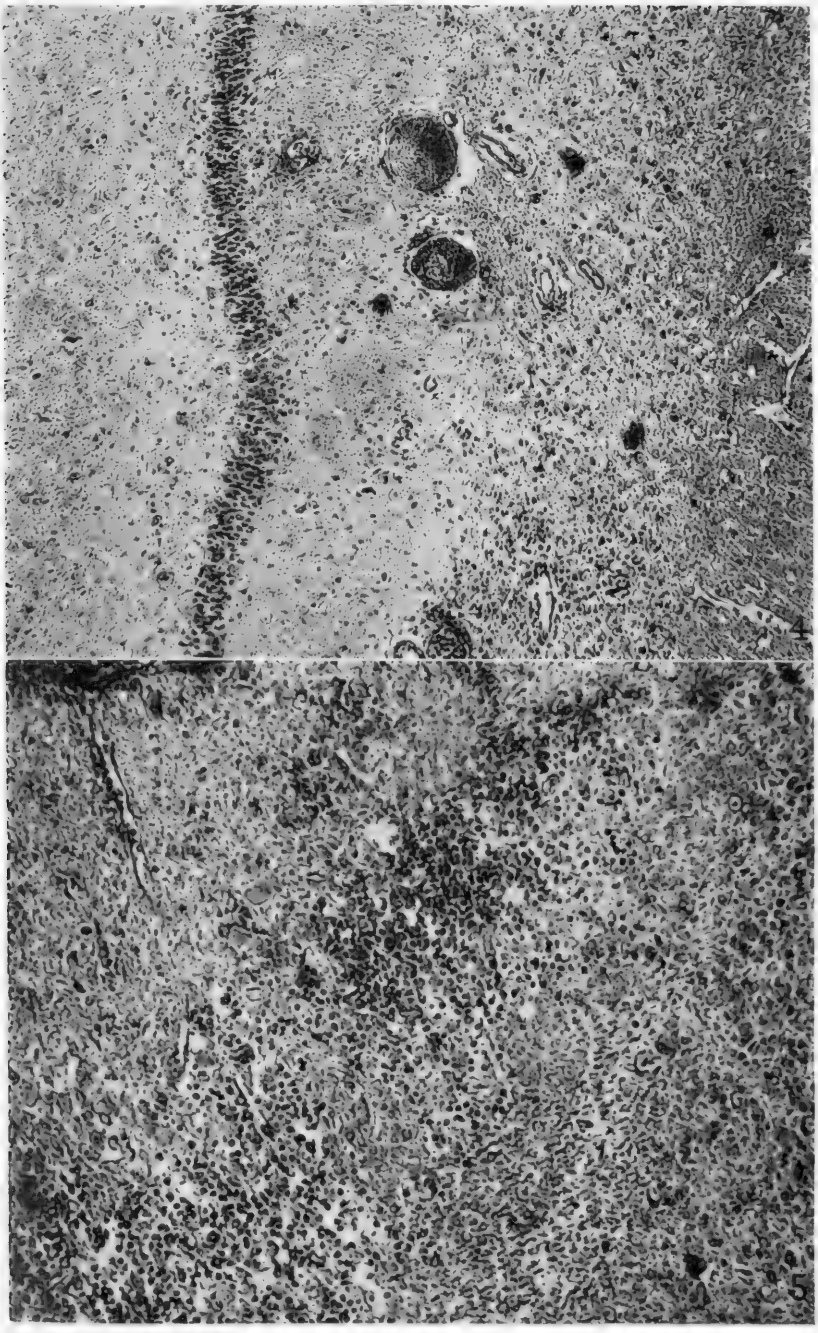
FIG. 7. Endothelial reaction in cortical meninges. Three nuclear inclusions, *a*, *b*, *c*. Zenker; Giemsa. × 850.

FIG. 8. Cortex showing perivascular infiltration. Zenker; eosin-methylene blue. × 130.



Photographed by Louis Schmidt.

(Rivers and Stewart: Virus III encephalitis.)



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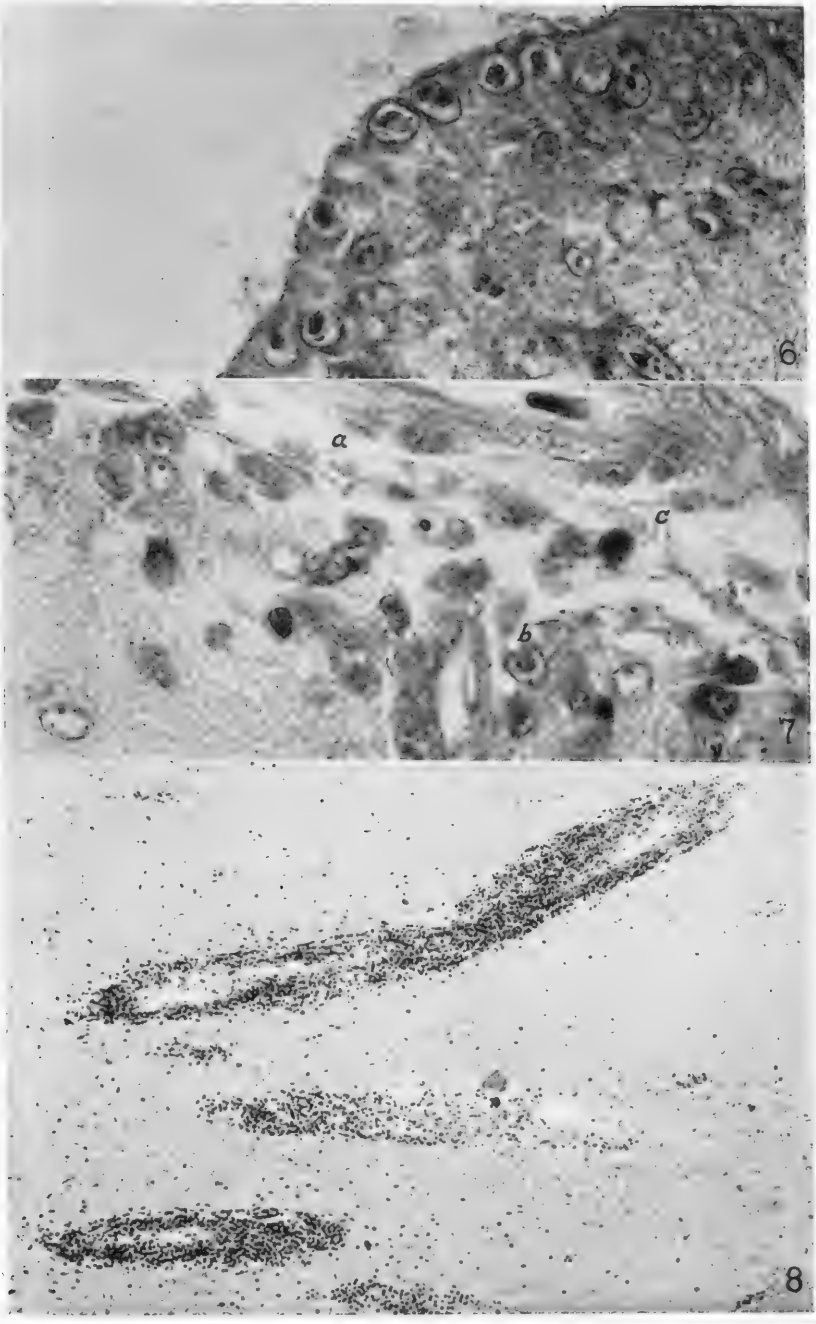

Photographed by Louis Schmidt.

(Rivers and Stewart: Virus III encephalitis.)

SURFACE TENSION OF SERA AS AFFECTED BY THE PRECIPITIN REACTION.

By SUSAN GRIFFITH RAMSDELL.

(*From the Research Institute of Cutaneous Medicine, Philadelphia.*)

(Received for publication, July 3, 1928.)

In a study of the surface tension changes in the blood of the guinea pig in anaphylactic shock, the author concluded[1] that the first lowering of the surface tension was probably due to the simple additive effect of the antigen serum, and that the significant changes were probably due to the effect of secondary tissue products such as cholesterol. But, if the basic reaction in anaphylaxis is a formation of a precipitate in the antigen serum by the cell-combined or circulating antibody, and if the essential effect upon the antigen serum is a denaturing one,—such as occurs in serum following heating, ageing, and shaking, by which the serum loses its capacity to lower the surface tension of a solution in saline,[2]—then one can explain this failure of the anaphylactic serum to show this characteristic change either by the assumption that precipitation plays no part in the phenomenon of shock, or that the guinea pig's serum is too weak in precipitin to afford an appreciable effect on surface tension, or that specific serum precipitation does not affect surface tension in the way that might be expected from that found for other precipitating or flocculating processes. For a study of this last assumption, a precipitating serum of high titre was chosen as furnishing the optimal conditions for any surface tension effect upon either the antibody or the antigen serum as a consequence of precipitation.

The sera of a normal rabbit and of a rabbit immunized against horse serum were studied for such surface tension changes as might occur as a consequence of the precipitin reaction. As antigen, a specially collected lot of normal horse serum was used. In making the readings, the serum in question was finally diluted to 1/10,000 for conformity

[1] Ramsdell, S. G., *J. Exp. Med.*, 1928, xlvii, 993.

[2] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575.

with previously reported immune serum values.[1,3] The du Noüy tensiometer was used for making the readings.

EXPERIMENTS.

I. Effect of the Precipitin Reaction upon the Surface Tension of the Immune Serum.

A mixture was made of 0.2 cc. of an immune rabbit serum, with a precipitin titre of 1/10,000, and 0.8 cc. of a 1/100 dilution of normal horse serum; this was incubated for 1 hour at 38°C., after which a final dilution of the mixture was made so that the resulting rabbit serum dilution was 1/10,000. Static surface tension readings showed a higher value by 2 dynes in this mixture of immune and antigen sera over that shown by the same dilution of the immune serum alone. When a control mixture of normal rabbit serum and antigen, and a dilution of normal rabbit serum alone were read for static surface tension values, there was a change of less than a dyne caused by the presence of the horse serum, and the difference between the static readings of the normal and immune serum mixtures was less than 2 dynes—a difference not significant of any effect of the antigen serum upon the antibody serum.

II. Effect of Various Dilutions of Antigen Serum upon the Static Reading of the Immune Serum.

Dilutions of horse serum from 1/10 to 1/100,000 were incubated in the water bath with a fixed amount of undiluted immune serum, in proportion of 0.9 cc. of the former to 0.1 cc. of the latter, for 30 minutes. A definite clouding was evident in the 1/10,000 tube. Dilutions were then made so that the immune serum was in a constant dilution of 1/10,000. There was a regular decrease of the static surface tension value with the increase of concentration of the horse serum from 1/100,000,000 to 1/10,000, but with only a difference of 3.5 dynes between the highest and the lowest, a value that might be attributed to a simple additive lowering effect of the horse serum.

This conclusion was confirmed by the control experiment where like mixtures and dilutions of normal rabbit serum and horse serum gave a difference of 2.1 dynes between the highest and lowest dilutions of the latter serum.

[3] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 707.

III. Effect of Precipitin on the Surface Tension of Antigen Serum.

Normal and immune sera, 0.1 cc., were incubated about 20 minutes at 40–50°C. with 1 cc. horse serum, 1/1,000. The immune serum gave a heavy turbidity. Both mixtures were then further diluted to a concentration of horse serum 1/10,000. The difference between the static readings was well within the limits of experimental error. When the experiment was repeated with the horse serum in a dilution of 1/100, which was that giving the maximum precipitate, the results gave even closer values.

Since the relatively great concentration of rabbit serum alone might constitute a factor for the lowering of surface tension in a mixture with antigen, advantage was taken of the high titre (1/40,000), which the immune serum now showed, to use a dilution of 1/20 of the latter in repeating the previous experiment.

The result was the same: the antigen showed no surface tension change that could be ascribed to a denaturing effect by the homologous immune serum.

IV. Effect of the Antigen-Antibody Reaction on Surface Tension in Function of Time.

The last part of Experiment III was repeated, with the difference that the sera were added directly to the horse serum dilution in two series of watch-glasses, and readings were taken at intervals: the resulting curves showed no significant difference in character.

CONCLUSIONS.

An attempt to find evidence, through the study of surface tension, using the du Noüy tensiometer, of a denaturing effect of the precipitin reaction upon either the antigen or the immune serum yielded entirely negative results.

ETIOLOGY OF OROYA FEVER.

XIII. Chemotherapy in Experimental Bartonella bacilliformis Infection.

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Plates 15 and 16.

(Received for publication, January 13, 1928.)

In the course of our studies on *Bartonella bacilliformis* infection in monkeys, we submitted several *Macacus rhesus*, in which experimental verrucous lesions had been induced by means of cultures, to treatment with chemicals which had proved therapeutically useful in spirochetal and leishmania infections. Salvarsan had already been recommended by Arce[1] in the treatment of malignant verruga in man. In our experiments salvarsan, neosalvarsan, bismuth lactate, esters of chaulmoogra oil, sodium gynocardate, neutroflavine, and urotropin were tested. It was not deemed suitable to test tartar emetic, since it is a drug of slow therapeutic action, and the experimental verrucous lesions in the monkey lead in any case to spontaneous retrogression.

Chemical Action in Vitro.

Although it was not expected that a relationship would be shown to exist between the action of the chemical when tested on *Bartonella in vitro* and the verrucous lesions *in vivo*, it was considered of interest to determine the direct effects of the chemicals on the bacilli. The substances to be tested were added directly to the culture media, and the cultures were incubated at 25°C. for a period of 13 days. The results are shown in Table I. Neutroflavine inhibited growth in 1:10,000,000 dilution. Formalin was almost as effective, and neosalvarsan, novasurol, and mercuric chloride were effective up to

[1] Arce, J., *An. Facultad Med. Lima*, 1918, i, No. 3, 21–53, 130–161; No. 4, 24–52.

TABLE I.

Growth-Inhibiting Properties in Vitro.

	Final concentration of substance in culture medium					
	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000	1:10,000,000
Bismuth albuminate	− (Turbid)	− (Turbid)	++++	++++	++++	++++
Tartar emetic	− (Turbid)	− (Clear)	−	+	++++	++++
Neosalvarsan	− (Clear, deep brown)	− (Brown)	− (Yellowish)	−	++	++++
Tryparsamide	± (Clear)	++++	++++	++++	++++	++++
Mercuric chloride	(Turbid)	− (Clear)	−	−	++++	++++
Novasurol	− (Clear)	−	−	−	++++	++++
Mercurochrome	− (Deep red, clear)	− (Deep red)	− (Eosin red)	++++ (Lt. eosin)	++++ (Tr. pink)	++++
Neutroflavine	(Turbid, deep gold)	(Turbid, gold)	− (Greenish yellow, clear)	− (Greenish)	− (Lt. green)	− (Tr. green)
Optochin	(Turbid, white)	− (Opalescent)	− (Clear)	+++	+++	+++
Sodium taurocholate	(Turbid, brownish)	?	+ (Clear)	+	+++	+++
Phenol	− (Opalescent)	?	+++	+++	+++	+++
Formalin	− (Clear)	−	−	−	±	
Lugol's solution	− (Clear)	++++	++++	++++	++++	++++

− = complete inhibition of growth.
++++ = no inhibition of growth.

1:100,000. Mercurochrome and tartar emetic required at least 1:10,000 concentration to prevent growth.

The first experiments were made on monkeys in which the cherry-red verrucous lesions on the abdominal skin and eyebrows had reached maximal size and had persisted in this state for several days. Blood cultures taken shortly before or at the time of first injection of the chemical into the circulation proved subsequently to be negative, and bits of excised nodules taken at the same time[2] showed few bacilli or even none at all by culture or in section. These last findings could not be known at the time of treatment, since the bacilli require 10 to 14 days to become evident in culture.

However, distinction between the ordinary or spontaneous regression of the nodules and the regression taking place after the use of chemicals, is entirely possible. The mature nodules undergo spontaneous regression slowly,[3] while in the animals given chemicals there occurred rapid loss of cherry-red color, usually in 24 to 48 hours after the first injection, followed by a still more rapid reduction in size most pronounced in the nodules located in the abdominal skin. At the expiration of 5 to 6 days small pale fibrous areas alone remained to indicate the site of the nodules, and in 10 to 14 days all vestiges had disappeared, the lesions of the eyebrows persisting somewhat longer than those of the abdomen. The protocols of these experiments follow.

Protocols.

Macacus rhesus 1-T, injected intravenously on Oct. 14, 1926, with 0.5 cc. of a mixture of:

4 cc. defibrinated blood (culture +++) from *M. rhesus* 54 (P. 5 strain[4]),

5 cc. culture of *Bartonella bacilliformis* (P. 5 strain) grown on leptospira medium for 72 hours,

5 cc. culture of *Bartonella bacilliformis* (P. 5 strain) grown for 6 days on blood agar slants.

In addition, 4 intradermal injections of the mixture were made on the left abdominal wall and 2 on the left eyebrow. Also nodular tissue freshly excised from *M. rhesus* 54 was applied to scarified areas on the right abdominal wall and right eyebrow.

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[2] Ether anesthesia was used in all the operations.

[3] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 455.

[4] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

Experimental nodules appeared on the left eyebrow in 14 days. Within a month large nodules were present on the abdomen at the sites of intradermal inoculation, and the scarified areas showed the characteristic miliary lesions. On Nov. 23 (40 days after inoculation) the hemoglobin was 35 per cent (Sahli), and the red cells 2,502,000, and there had been no fever. 0.05 gm. of salvarsan was given intravenously. Within the following week the nodules grew small and became paler. On Dec. 3 a second injection of 0.05 cc. salvarsan was administered. Within the week the nodules had become very small and pale, the erythrocytes rose to 5,600,000 and the hemoglobin to 80 per cent. When the animal was sacrificed on Dec. 13, *Bartonella bacilliformis* could not be detected in the nodular tissue either microscopically or by culture, and blood, lymph nodes, and spleen also failed to yield cultures. Sections of the nodules showed fibrous tissue.

Macacus rhesus 2-T. Inoculated at the same time as No. 1-T, and in the same manner, except that the eyebrows were spared. On Oct. 19 the culture titer of the blood was 1:100,000. On Nov. 15, or 32 days after the inoculation, the animal showed large mature subcutaneous nodules on the left abdominal wall and numerous red miliary lesions on the scarified areas on the right side (Fig. 1). Blood cultures made on this day later proved to be negative. A mixture of 1 cc. of 1 per cent bismuth lactate, 1 cc. of 1 per cent neutroflavine, and 1 cc. of 1 per cent urotropin was injected intravenously. The temperature rose to 105.2°F. on the following day (Nov. 16), but the animal appeared well. On Nov. 23, a second injection of the same mixture was given. The nodules soon became bluish, smaller in size, and continued to diminish in volume after the second dose of the drugs (Fig. 2). On Dec. 3 a third injection was given. During the following 9 days the nodules became very small and pale (Fig. 3). Nodules and spleen removed on Dec. 16 did not yield *Bartonella bacilliformis* in culture.

Macacus rhesus 3-T, inoculated in same manner as *Macacus* 2-T. By Nov. 24 the abdomen showed large, mature subcutaneous nodules (Fig. 4), and large bluish red nodules on each leg where the injections had been made into the saphenous veins. A general miliary eruption was also present on the abdomen (Fig. 5). The hemoglobin was 45 per cent (Sahli), and the red blood cells 3,960,000. No fever. A suspension of one of the nodules yielded cultures of *Bartonella bacilliformis* in a dilution of 1:1,000, but the blood proved negative, although the titer had been 1:100,000 on Oct. 19 and Nov. 3. At this time (*i.e.*, on Nov. 24, 41 days after inoculation), 1 cc. of moogrol[5] was given intravenously. Within 48 hours the nodules had become bluish in color and somewhat smaller. Fig. 6 shows the appearance of the nodules 9 days after the first treatment. On Dec. 3 another injection of 1 cc. of moogrol was given. The temperature rose on the 2 days following to 104.4–104.6°F. By Dec. 13 the nodules had contracted considerably (Fig. 7). The spleen was negative for culture on Dec. 16, and all lesions had disappeared by Dec. 30. The blood culture was negative a week later.

[5] Burroughs Wellcome and Company's preparation of the esters of chaulmoogra oil.

Macacus rhesus 4-T was inoculated intravenously on Nov. 24, 1926, with 1 cc. of a mixture of cultures of *Bartonella bacilliformis*, besides which a suspension of an abdominal nodule of *M. rhesus* 3-T was given intravenously and introduced intradermally into both eyebrows and the abdominal skin. On Dec. 21 the blood cultures were positive for *Bartonella bacilliformis* in a 1:100,000 dilution. The nodules were fully developed by Dec. 28, 34 days after inoculation (Figs. 8 and 11), when the first intravenous injection of bismuth lactate, proflavine, and urotropin was given (a mixture of 1 cc. of a 1 per cent solution of each). Blood cultures were negative at this time. The temperature rose to 105°F. on the day following the treatment, and the nodules had already shrunk and become paler. A double dose was given on Dec. 30, and again on Jan. 3, 1927. No febrile reactions. Blood culture was negative on Jan. 5, 1927. The hemoglobin was 78 per cent (Sahli), and the erythrocytes 4,400,000. Cultures made from nodules and lymph nodes on Jan. 10, 1927, were negative. The lesions regressed rapidly (Figs. 9 and 12), and only fibrous traces remained on Jan. 28, 1927 (Figs. 10 and 13).

Macacus cynomolgus 5-T, inoculated intradermally with a suspension of nodular tissue from *M. rhesus* 1-S[6] into the right eyebrow and the abdominal skin on Dec. 7, 1926. This animal remained afebrile but developed large cherry-red nodules on eyebrows and abdomen by Dec. 27. The blood culture titer was 1:10 on Jan. 6, 1927. On Jan. 8 and 14, 1927, or 32 and 38 days after inoculation, an intravenous injection was made of 1 cc. of 5 per cent bismuth lactate, 1 cc. of 5 per cent urotropin, and 1 cc. of 1 per cent neutroflavine. Figs. 14 and 15 show the appearance of the lesions on Jan. 7. The nodules began to shrink in the following week and within 2 weeks had become small and fibrous. The appearance on Feb. 8 is shown in Figs. 16 and 17.

The next step was to test the action of the chemicals on the appearance and development of the nodules in instances in which the chemicals were administered *before* the lesions reached full development, *i.e.*, 2 to 3 weeks after the inoculation of the infective material into the skin, while the lesions were growing in size daily. Under these circumstances the drugs failed to influence the progress of the lesions, which in one instance attained unusually large proportions (Figs. 19, 20). The usual cherry-red color developed without hindrance, and the scarified areas became covered with the characteristic deep red miliary nodules. As in untreated animals, the skin of the lower half of the abdomen became edematous.

[6] Noguchi, H., *J. Exp. Med.*, 1928, xlvii, 821.

Protocols.

Macacus rhesus 6-T, inoculated intravenously (2 cc.) and intradermally on Dec. 28, 1926, with a mixture of cultures of *Bartonella bacilliformis* and a suspension of nodular tissue of *M. rhesus* 4-T. The nodules were well advanced on Jan. 24, 1927, 27 days after the inoculation, when intravenous injection of a mixture of bismuth, urotropin, and proflavine (1 cc. of 1 per cent proflavine, 1 cc. of 5 per cent urotropin, and 1 cc. of 5 per cent bismuth lactate) was begun. Blood taken just before the treatment yielded cultures in a dilution of 1:10. No change was observed in the nodules after the first injection. The second injection, given on Jan. 26, 1927, was followed by slight diminution in the size of the nodules on the eyebrows while the abdominal lesions continued to enlarge.

Macacus rhesus 7-T, inoculated by scarification and intradermal injection on Mar. 8, 1927, with a suspension of the nodule from *M. rhesus* 3-A, which had been infected by means of the L_5 strain of *Bartonella bacilliformis*.[7] Small nodules were present 20 days after inoculation (Fig. 18), when the animal was given the first intravenous injection of 0.1 gm. of neosalvarsan. The injection was repeated 2 days later. The nodules continued to grow gradually and within 2 weeks they attained unusually large dimensions (Figs. 19 and 20). Certainly no inhibitory action was apparent.

Macacus rhesus 8-T. This animal was inoculated in the same manner and on the same date as the foregoing monkey. Nodules had appeared by Mar. 28 (Fig. 21), when an intravenous injection of 2 cc. of 3 per cent sodium gynocardate and an intramuscular injection of 0.15 cc. of chaulmestrol[8] were given. This was followed 2 days later by an injection of 3 cc. of the sodium gynocardate and an injection of 0.2 cc. of chaulmestrol. No inhibitory effect upon the development of the cutaneous lesions was apparent and the large nodules are shown in Figs. 22 and 23.

SUMMARY.

The therapeutic effect of several antiparasitic chemicals on experimental verruga peruana is described. The drugs were administered by intravenous injection according as the nodules (1) were already developed to an approximate maximum, or (2) were still in the active period of growth.

The effect of the drugs was different under the two circumstances of

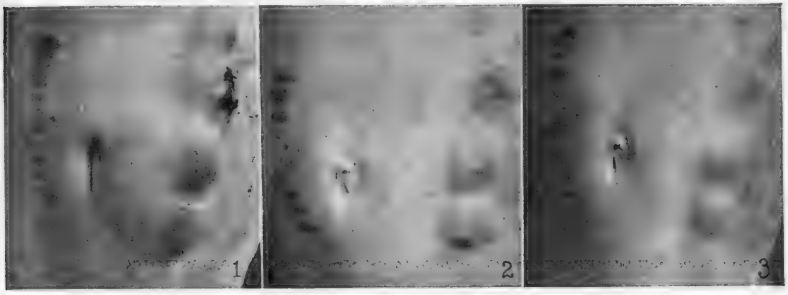
[7] Noguchi, H., *J. Exp. Med.*, 1928, xlvii, 219.

[8] The name given by the Winthrop Chemical Company to their preparation of the esters of chaulmoogra oil, of which the Company kindly furnished a sample.

their administration. When they were given after the maturity of the nodules they hastened the regressive process, but when given during active growth of the lesions no action whatever was detected.

Bartonella bacilliformis in culture is acted upon injuriously by a number of the chemicals employed in the therapeutic tests, the most active being formalin and neutroflavine.

M. rhesus 2-T.



Before treatment (32 days after inoculation).

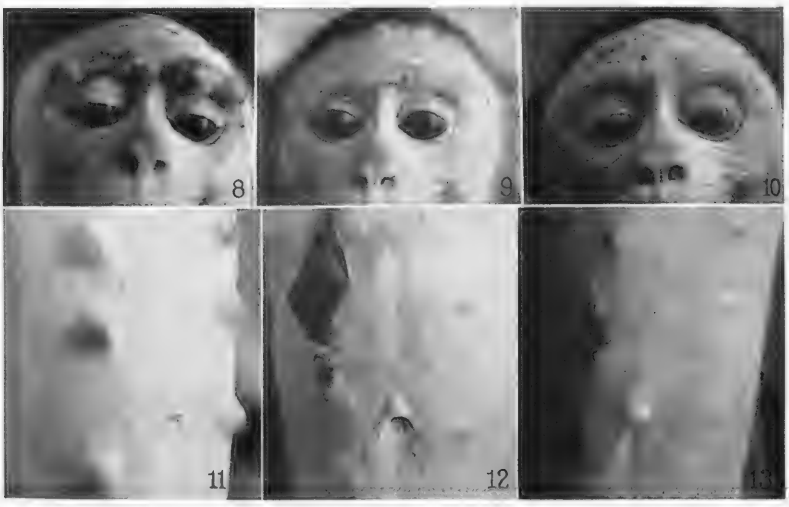
17 days after treatment. 25 days after treatment. (One nodule removed for study 32 days after inoculation.)

M. rhesus 3-T.

Before treatment. Nodular and miliary eruptions 41 days after inoculation.

9 days after treatment. 19 days after treatment. (One nodule removed for study 41 days after inoculation.)

M. rhesus 4-T.



Before treatment. Nodules on eyebrows and abdomen 34 days after inoculation.

13 days after treatment. 38 days after treatment. (One nodule removed for study 34 days after inoculation.)

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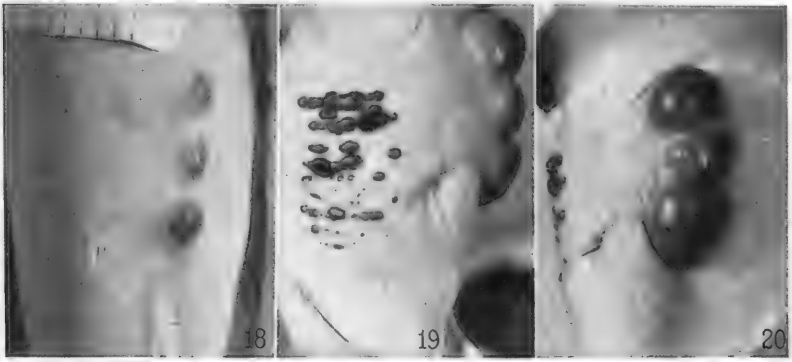
M. cynomolgus 5-T.

Before treatment (32 days after inoculation).

31 days after treatment was begun.

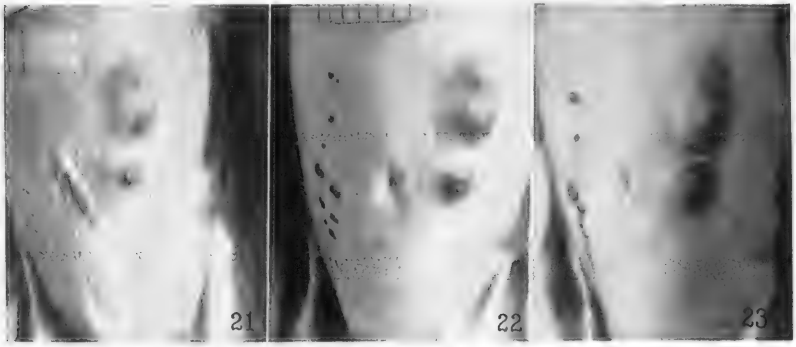
M. rhesus 7-T.



Before treatment (20 days after inoculation).

15 days after treatment was begun.

M. rhesus 8-T.



Before treatment (20 days after inoculation).

8 days after first treatment.

15 days after treatment was begun.

(Noguchi: Etiology of Oroya fever. XIII.)

UNIV. OF

THE EFFECT OF SODIUM CHLORIDE ON THE CHEMICAL CHANGES IN THE BLOOD OF THE DOG AFTER OBSTRUCTION OF THE CARDIAC END OF THE STOMACH.

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After obstruction of the pyloric end of the stomach, there is a rapid fall in the blood chlorides, an increase in the CO_2 combining power, and a rise in the non-protein nitrogen (1). With the vomiting which necessarily follows pyloric obstruction some chloride is lost in the vomitus. In attempting to eliminate the loss of chloride in this manner, the blood chemistry of the dog was studied after ligation of the cardiac end of the stomach (2). It was found in such experiments, that there is a rapidly developing toxemia with marked rise in non-protein nitrogen and usually some fall in the chlorides. The toxemia is even more marked than that following pyloric and high intestinal obstruction.

It has been shown that solutions of sodium chloride will prevent or relieve the characteristic toxemia of experimental pyloric and intestinal obstruction (3). The experiments reported herewith have been made to determine if solutions of sodium chloride have a similar effect on the toxemia of cardiac obstruction.

Method.

The animals have been kept in metabolism cages during the experiments. No food was given for 24 hours preceding operation and no food or water during the course of the experiments. All ligations were done under ether anesthesia with aseptic technique. Local anesthesia was employed when releasing the obstruction. The obstruction was made by tying tape around the cardiac end of the stomach as closely as possible to the lower end of the esophagus.

The non-protein nitrogen of the blood was determined by the method of Folin and Wu (4), the CO_2 combining power by the method of Van Slyke and Cullen (5),

the urea nitrogen by the Van Slyke and Cullen modification of the Marshall method (6), and the chlorides on the tungstic acid filtrate as suggested by Gettler (7). The nitrogen in the urine was determined by a micro Kjeldahl method (8) and the chlorides by a modified Volhard-Arnold method.

TABLE I.

Effect of Simple Obstruction of Cardiac End of Stomach.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Amount	Chlorides		Nitrogen		
		mg.	*mg.*	*mg.*	*vol. per cent*	*cc.*	*per cent*	*gm.*	*per cent*	*gm.*	
4–48	0	33.0	16.1	470	40.0	100	0.27	0.27	0.46	0.46	No treatment
(wt.	1	59.5	30.1	450	40.0	225	0.34	0.77	1.38	3.10	
17 kg.)	2	146.0	87.6	460	30.5	455	0.75	3.41	0.96	4.37	
	3					140	1.00	1.40	0.62	0.87	
4–49	0	27.7	12.6	520	36.2	105	0.10	0.11	0.21	0.22	No treatment
(wt.	1	32.6	11.2	460	30.5	300	1.03	3.10	2.00	6.0	
17 kg.)	2	51.8	27.9	420	32.4	500	0.64	3.20	0.40	2.0	
	3	68.8	28.7	530	38.1	485	0.47	2.28	2.00	9.7	
	4					320	0.50	1.60	1.50	4.80	
4–51	0	28.9	9.8	470	40.0						No treatment
(wt.	1	31.9	11.8	450	43.8	535	1.40	7.44	1.32	7.06	
20 kg.)	2	82.5	46.9	440	34.3	380	0.68	2.59	1.44	5.47	
	3	139.0	84.1	400	32.4	420	0.65	2.73	0.82	3.44	
	4					105	0.48	0.50	0.62	0.65	

OBSERVATIONS.

Table I shows the characteristic findings in blood and urine after obstruction of the cardia. There is a rapid rise in non-protein nitrogen, very little change in the CO_2 combining power, and some tendency toward a fall in chlorides. A marked diuresis occurs with a high nitrogen and chloride output. One animal died before the 3rd day and two before the 4th day.

In Table II are recorded the findings in three dogs similarly obstructed and given 40 cc. per kilo body weight of 1 per cent sodium

TABLE II.

Obstruction of Cardiac End of Stomach.

Treatment with 1 Per Cent Sodium Chloride Subcutaneously.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Amount	Chlorides		Nitrogen		
		mg.	*mg.*	*mg.*	*vol. per cent*	*cc.*	*per cent*	*gm.*	*per cent*	*gm.*	
5–47	0	33.7	17.5	520	52.0	50	0.78	0.39	1.44	0.72	Operation. 40 cc. NaCl per kg. injected
(wt. 11 kg.)	1	24.0	11.9	480	36.2	250	0.95	2.38	0.62	1.60	40 cc. NaCl per kg. injected
	2	29.0	16.1	580	32.4	870	0.63	5.50	1.04	9.2	" "
	3	23.4	10.5	550	38.1	285	0.77	2.20	0.96	2.8	" "
	4	28.2	16.1	620	47.5	215	0.84	1.80	1.10	2.4	" "
	5	40.0	14.7	600	38.1	205	0.80	1.64	1.32	2.8	" "
	6	30.0	17.5	650	41.9						" "
	7	27.3	15.4	690	40.0	135	1.20	1.62	0.46	0.64	No injection
	8	27.5	18.9	600	45.7	110	1.06	1.17	1.02	11.12	" "
	9	29.4	18.2	670	34.3						Injection given
	10	25.9	14.0	680	41.9						No injection
	11	31.6	20.3	640	34.3	210	1.10	2.31	1.50	3.2	" "
	12	68.3	35.0	640	34.3						Injection given
	13	36.9	20.3	660	38.1	225	0.95	2.14	1.56	3.6	" "
	14					100	0.93	0.93	1.23	1.23	No injection
	15	39.0	25.9	690	32.4	85	1.70	1.45	1.20	1.08	" "
	17	46.5	23.8	780	32.4						" "
	18					170	1.69	2.87	1.10	1.9	Injection given
	19	48.5	21.7	630	30.5						" "
	21					130	0.61	0.79	1.44	1.9	No injection
	22	30.6	20.1	630	28.7						Injection given
	23					190	1.58	3.02	1.84	3.5	No injection
	24	28.9	17.3	610	30.5	79	1.83	1.43	1.68	1.3	Injection given
	26	25.2	15.4	590	25.6	180	1.85	3.33	1.34	2.0	" "
	28	27.0	13.5	620	32.4	65	1.08	0.70	1.41	0.9	" "
	29					100	1.80	1.79	1.41	1.4	No injection
	30	29.1	17.8	540	41.9						Injection given
	31					140	1.70	2.31	1.50	2.1	No injection
	32	26.3	11.2	540	34.3	170	1.60	2.72	1.25	2.1	Injection given
	33					200	1.45	2.90	1.37	2.7	" "

TABLE II—*Continued.*

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Amount	Chlorides		Nitrogen		
		mg.	*mg.*	*mg.*	*vol. per cent*	*cc.*	*per cent*	*gm.*	*per cent*	*gm.*	
5–47	34	51.0	24.8	540	41.9	215	0.53	1.14	1.72	3.7	No injection
(continued)	35					175	0.52	0.91	1.60	2.8	Injection given
	36	58.3	35.0	540	38.1	90	0.35	0.32	1.74	1.6	Died
5–48 (wt. 9.6 kg.)	0	36.1	21.7	450	30.5						Operation. 40 cc. 1 per cent NaCl per kg. injected
	1	24.4	9.8	470	32.4	660	0.61	4.0	0.28	1.9	40 cc. 1 per cent NaCl per kg. injected
	2	33.3	14.0	550	29.6	470	0.72	3.4	0.28	1.3	" "
	3	24.4	12.6	520	32.4	195	0.77	1.5	0.76	1.5	" "
	4	22.8	14.7	570	32.4	155	1.03	1.6	1.00	1.5	" "
	5	32.4	16.4	590	28.7	135	1.21	1.6	1.32	1.9	" "
	6	34.5	25.2	620	30.5						" "
	7	39.1	25.2	590	29.6						No injection
	8	43.2	32.2	560	29.6	155	1.36	2.1	1.20	1.9	Injection given
	9	45.7	27.9	570	30.5						" "
	10	27.0	17.5	560	30.5						No injection
	11	30.0	16.1	540	27.5						" "
	12	50.5	21.7	640	27.5	110	1.18	1.3	1.44	1.6	" "
	13	44.2	32.9	550	30.5						Injection given
	15	40.0	31.5	550	28.7	150	1.69	2.5	1.32	2.0	No injection
	17	46.3	20.1	530	28.7						" "
	18					125	0.79	1.0	1.50	1.9	" "
	19	48.7	27.3	540	28.7						Injection given
	21					80	0.96	0.8	1.64	1.3	No injection
	22	41.7	22.9	540	25.6						Injection given
	23					80	1.50	1.2	1.60	1.3	No injection
	24	42.2	25.7	570	23.0	120	1.61	1.9	1.88	2.3	Injection given
	26	22.9	15.9	550	25.6	130	1.86	2.4	1.64	2.1	" "
	27										No injection
	28	22.2	10.3	550	27.7	155	1.32	2.1	1.07	1.7	Injection given
	29					160	1.34	2.2	1.25	2.0	No injection
	30	26.1	10.7	500	28.7						Injection given
	31					200	1.41	2.8	1.08	2.2	" "
	32	23.0	9.3	490	30.5	130	0.97	1.3	0.93	1.2	" "
	33					110	2.10	2.3	1.25	1.4	" "
	34	20.4	8.9	490	27.7	135	1.27	1.7	0.97	1.3	No injection

TABLE II—*Concluded.*

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Amount	Chlorides		Nitrogen		
		mg.	*mg.*	*mg.*	*vol. per cent*	*cc.*	*per cent*	*gm.*	*per cent*	*gm.*	
5–48	35					95	1.28	1.2	0.98	1.0	Injection given
(contin-	36	24.2	14.5	500	34.3	90	0.82	0.7	1.56	1.4	" "
ued)	38	22.8	9.8	560	30.5						" "
	40					200	1.16	2.3	1.98	4.0	" "
	43					210	0.88	1.9	2.34	5.0	No injection
	45	24.0	10.3	520	32.4						Died
5–55 (wt. 18 kg.)	0	25.0	15.4	470	32.4	65	1.05	0.68	0.66	0.42	Operation. 40 cc. 1 per cent NaCl per kg. injected
	1	25.2	12.6	470	37.2	165	0.99	1.62	0.96	1.63	40 cc. 1 per cent NaCl per kg. injected
	2	22.5	15.4	520	37.2	225	1.15	2.59	1.04	2.34	" "
	3	25.2	14.0	510	38.1	480	1.50	7.20	0.86	4.13	" "
	4	27.7	10.5	510	38.1	115	1.30	1.50	0.76	0.91	" "
	5	22.5	14.0	540	34.3						" "
	6	26.3	10.5	560	36.2	670	1.34	8.98	0.59	3.95	" "
	7	23.3	8.4	600	36.2	220	1.62	3.56	1.04	2.10	" "
	8					225	1.50	3.38	1.10	2.53	No injection
	9	25.2	14.9	580	31.5						Injection given
	10	21.6	12.6	610	35.3	130	1.92	2.50	2.68	3.50	No injection
	11	30.0	21.0	550	26.8	80	1.91	1.53	2.04	1.63	Injection given
	13	23.6	10.3	560	32.4	100	1.96	1.96	1.88	1.88	" "
	14					190	1.93	3.67	1.94	3.69	No injection
	15	21.8	8.4	530	30.5	110	1.94	2.14	1.72	1.90	Injection given
	16					80	1.96	1.57	1.56	1.25	No injection
	17	30.9	15.9	470	30.5						Injection given
	18					160	1.93	3.09	2.22	3.6	No injection
	19	28.0	15.9	480	35.3	120	1.86	2.23	1.34	1.61	Injection given
	20					440	1.40	6.16	0.72	3.17	" "
	21	75.8	43.4	450	36.2	125	1.04	1.31	0.65	0.81	No injection
	22					130	1.05	1.37	0.77	1.0	Injection given
	23	24.8	9.3	430	38.1	160	1.69	2.71	1.68	2.69	" "
	25	23.4	6.5	440	36.2						" "
	27					320	1.18	3.78	0.73	2.34	" "
	29	26.8	9.3	470	38.1						No injection
	30					180	1.50	2.70	1.45	2.61	
	32	27.0	7.4	450	34.3						Died

chloride subcutaneously at varying intervals. There is no rise in non-protein or urea nitrogen. The CO_2 combining power shows little

TABLE III.

Obstruction of Cardiac End of Stomach.

Treatment with 2 Per Cent Glucose Subcutaneously.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Amount	Chlorides		Nitrogen		
		mg.	*mg.*	*mg.*	*vol. per cent*	*cc.*	*per cent*	*gm.*	*per cent*	*gm.*	
5–60 (wt. 13 kg.)	0	29.4	17.3	420	28.7						Operation. 40 cc. of 2 per cent glucose per kg. injected
	1	33.0	24.3	410	29.6	700	0.88	6.13	1.30	9.1	40 cc. of 2 per cent glucose per kg. injected
	2	123.0	73.8	380	20.2	230	1.15	2.65	0.63	1.5	" "
	3					140	0.70	0.98	1.36	1.9	Died preceding night
5–61 (wt. 15 kg.)	0	34.9	23.4	520	38.1						Operation. 40 cc. 2 per cent glucose per kg. injected
	1	44.4	35.0	420	27.7	800	0.62	4.96	2.08	16.8	40 cc. of 2 per cent glucose per kg. injected
	2	138.0	66.9	400	29.6	400	0.84	3.36	0.47	1.9	" "
	3	188.0	83.6	380	18.3						" "
	4					250	0.53	1.33	0.40	1.0	Died
5–62 (wt. 15.5 kg.)	0	45.6	22.9	460	35.3						Operation. 40 cc. 2 per cent glucose per kg. injected
	1	39.4	21.5	400	25.8	900	1.31	11.8	2.5	22.5	40 cc. of 2 per cent glucose per kg. injected
	2	63.5	29.4	390	29.6	250	1.11	2.8	0.75	1.9	" "
	3	107.0	61.2	360		350	0.45	1.6	2.04	7.1	" "
	4					70	0.33	0.3	1.07	0.8	Dead

change; the chlorides tend to remain higher than normal. All three animals show the initial diuresis with high nitrogen and chloride

excretion, followed by a period of normal output. Near the end there is again an increased excretion of water, salt, and nitrogen. The

TABLE IV.

Obstruction of Cardiac End of Stomach.

10 Per Cent Sodium Chloride Introduced Directly into Jejunum.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Amount	Chlorides		Nitrogen		
		mg.	*mg.*	*mg.*	*vol. per cent*	*cc.*	*per cent*	*gm.*	*per cent*	*gm.*	
5–63 (wt. 17 kg.)	0	21.1	10.3	460	22.1						Operation. 4 cc. 10 per cent NaCl per kg. in jejunum
	1	34.3	14.5	540	30.5	190	0.96	1.82	0.4	0.76	4 cc. 10 per cent NaCl per kg. in jejunum
	2	34.1	17.8	580	24.0	260	0.89	2.32	1.4	3.64	" "
	3	53.5	40.6	620	27.7	310	0.72	2.23	2.1	6.50	" "
	4					55	1.79	0.99	1.2	0.7	Dead
5–64 (wt. 17 kg.)	0	61.5	28.0	540	34.3						Operation. 4 cc. 10 per cent NaCl per kg. in jejunum
	1	44.4	19.6	450	36.2	630	1.01	6.4	1.30	8.2	4 cc. 10 per cent NaCl per kg. in jejunum
	2	45.0	20.1	510	34.3	270	0.66	1.8	1.94	5.3	" "
	3	43.2	23.4	560	34.3	360	0.62	2.2	2.22	8.0	" "
	4					20	1.60	0.3	2.42	0.48	Dead
5–65 (wt. 18 kg.)	0	29.1	12.1	510	38.1						Operation. 4 cc. 10 per cent NaCl per kg. in jejunum
	1	50.4	25.2	460	29.6	1030	1.01	10.1	0.60	6.2	4 cc. 10 per cent NaCl per kg. in jejunum
	2	52.6	21.0	580	24.9	350	0.80	2.8	1.11	3.9	" "
	3	50.4	34.6	580	29.4	660	0.95	6.3	1.14	7.5	" "
	4	74.3	43.9	660	25.8	70	1.72	1.21	1.72	1.2	" "

animals survived 32, 36, and 45 days. At autopsy in each animal, the tape had cut through, thus reestablishing continuity. As a con-

trol on these experiments three animals were given 40 cc. per kilo of 2 per cent glucose. These show the changes characteristic of untreated animals with rapid death. The diuresis and salt and nitrogen excretion are even more marked than in untreated animals.

To determine the effect of sodium chloride given with a minimum of water on the toxemia of cardiac obstruction, three dogs were given

TABLE V.

Obstruction and Release of Cardiac End of Stomach.

No Treatment.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Amount	Chlorides		Nitrogen		
		mg.	*mg.*	*mg.*	*vol. per cent*	*cc.*	*per cent*	*gm.*	*per cent*	*gm.*	
5–44	0	32.1	19.6	510	36.2	65	0.72	0.51	1.23	0.86	Cardiac end obstructed
(wt.	1	49.2	25.2	500	33.4	50	0.10	0.05	1.20	0.60	" " "
15 kg.)	2	87.0	51.8	520	28.7	200	0.60	1.2	0.96	1.90	Obstruction released
	3	111.0	56.8	460							Died
5–46	0	25.2	11.2	500	35.3	60	0.37	0.22	1.16	0.70	Cardiac end obstructed
(wt.	1	35.3	27.3	490	32.4	555	0.41	2.30	0.30	1.74	
9 kg.)	2	91.5	45.6	470	28.7	225	0.60	1.35	0.78	1.80	Obstruction released
	3	134.0	65.2	430	12.4	25	0.17	0.05	0.62	0.20	Died
5–45	0	22.9	14.7	500	38.1	45	0.16	0.30	1.50	0.8	Cardiac end obstructed
(wt.	1	29.0	11.4	450	32.4	750	0.8	6.0	0.54	4.1	
16 kg.)	2	129.0	62.3	450	28.7	285	0.8	2.32	0.55	1.6	Obstruction released
	3	230.0	111.0	420	21.9	65	0.02	0.14	1.0	0.65	Died

4 cc. per kilo of 10 per cent sodium chloride directly into the jejunum, through a tube introduced when the cardia was obstructed (Table IV). These animals died in approximately the same time as untreated animals, but showed much less rise in the non-protein nitrogen than untreated animals.

Several experiments were made to determine the effect of sodium chloride after obstruction and subsequent release of the cardia. In

TABLE VI.

Obstruction and Later Release of Cardiac End of Stomach. Treatment with 1 Per cent Sodium Chloride Solution Subcutaneously.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	
		mg.	*mg.*	*mg.*	*vol. per cent*	
5–41	0	34.1	15.4	520	45.7	Cardiac end of stomach obstructed
	1	34.1	15.4	490	34.3	
	2	40.0	21.0	510	32.4	Obstruction released. 40 cc. of 1 per cent NaCl per kg. injected
	3	75.0	45.5	560	28.7	
	4	34.9	8.4	510	29.6	40 cc. of 1 per cent NaCl per kg. injected
	5	28.0	16.1	560	28.7	40 " " 1 " " " " " "
	6	21.1	9.8	500	20.0	40 " " 1 " " " " " "
	7	26.1	9.8	600	21.9	40 " " 1 " " " " " "
	8	31.4	16.8	650	18.1	Died. Autopsy showed no cause for death
5–42	0	26.5	10.5	470	36.2	Cardiac end of stomach obstructed
	1	36.1	18.9	460	36.2	
	2	61.5	33.6	450	40.0	Obstruction released. 40 cc. 1 per cent NaCl per kg. injected
	3	32.3	14.0	510	36.2	
	4	22.8	7.0	470	41.9	40 cc. 1 per cent NaCl per kg. injected
	5	25.9	10.5	530	36.2	40 " 1 " " " " " "
	6	30.6	14.7	530	36.2	40 " 1 " " " " " "
	7	28.0	14.0	540	39.0	40 " 1 " " " " " "
	8	23.8	14.0	540	34.3	40 " 1 " " " " " "
	9	25.2	9.8	570	36.2	40 " 1 " " " " " "
	10	27.8	8.4	530	45.7	Normal
5–66	0	41.6	15.4	460	36.2	Cardiac end of stomach obstructed
	1	46.8	23.8	460	32.4	
	2	115.0	63.5	450	27.7	Obstruction released. 40 cc. 1 per cent NaCl per kg. injected
	3	38.1	19.2	490		
	4	25.7	11.2	540	50.2	40 cc. 1 per cent NaCl per kg. injected
	5	25.3	10.3	600	52.0	Normal

three animals, the tape was removed 48 hours after the cardia had been ligated. All died within 48 hours after the release. The non-protein nitrogen continued to rise after the ligature was removed (Table V).

In three animals 40 cc. of 1 per cent sodium chloride was injected subcutaneously each day after the removal of the obstruction (Table VI). In each instance the non-protein nitrogen, which had begun to rise before the ligature was removed, promptly fell to normal and

TABLE VII.

Obstruction and Later Release of Cardiac End of Stomach. Treatment with 2 Per Cent Glucose Solution Subcutaneously.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	
		mg.	*mg.*	*mg.*	*vol. per cent*	
5–69	0	21.2	5.6	440	34.3	Cardiac end of stomach obstructed
	1	40.6	16.8	400	30.5	
	2	40.8	15.9	420	32.4	Obstruction released. 40 cc. 2 per cent glucose per kg. injected
	3	62.5	27.1	370	25.8	40 cc. 2 per cent glucose per kg. injected
	4	71.3	37.4	370	27.7	40 " 2 " " " " " " "
	5	123.0	47.5	400		40 " 2 " " " " " " "
	6	146.0	55.6	330		Died
5–72	0	27.5	10.7	430	36.2	Cardiac end of stomach obstructed
	1	29.4	14.0	500	34.3	
	2	97.3	48.1	460	29.6	Obstruction released. 40 cc. 2 per cent glucose per kg. injected
	3	152.0	74.3	460	38.1	40 cc. 2 per cent glucose per kg. injected Died
5–73	0	40.0	9.3	410	36.2	Cardiac end of stomach obstructed
	1	35.7	17.3	310	41.9	
	2	103.0	51.4	300	28.7	Obstruction released. 40 cc. 2 per cent glucose per kg. injected
	3	163.0	81.3	440	47.5	40 cc. 2 per cent glucose per kg. injected. Died

remained so. Two animals recovered. One died on the 8th day, autopsy revealing no cause for death.

As a control on this last group, three animals were similarly obstructed and released and given 2 per cent glucose solutions subcutaneously after the removal of the obstruction (Table VII). One

animal lived for 6 days, with a gradual increase in non-protein and urea nitrogen. This animal also had a low blood chloride. Two animals died in less than 96 hours with the changes characteristic of untreated animals.

DISCUSSION.

These experiments emphasize the very marked toxemia which develops after obstruction of the cardiac end of the stomach of the dog. The changes in the blood chloride are small as compared with those observed after pyloric obstruction. The administration of 1 per cent sodium chloride prevented entirely the toxemia and prolonged life indefinitely. This effect is not due to fluid alone as the same amount of 2 per cent glucose solution does not have any effect on the development of the toxemia. The chloride in the blood of animals so treated is lower than in untreated animals without an increase in chloride excretion.

Sodium chloride solution is of the greatest value in the treatment of the toxemia after it has developed if the obstruction is surgically released. Simple release and treatment with glucose solution do not change the course of the toxemia. The results obtained in this series are similar to those observed in the treatment of the toxemia resulting from obstruction of the upper gastrointestinal tract at other levels.

SUMMARY.

A study is reported of the effect of different methods of treatment on the toxemia of cardiac obstruction.

The average duration of life of untreated dogs is 3 days. Three dogs treated with 1 per cent salt solution subcutaneously lived 32, 36, and 45 days respectively without developing a toxemia. 2 per cent glucose similarly given, does not alter the course of the toxemia. Concentrated salt solution in small quantities given directly into the jejunum prevents the marked rise in non-protein nitrogen but does not materially prolong life.

Release of the obstruction does not change the course of the toxemia in untreated animals. The subcutaneous injection of 1 per cent sodium chloride solution after release of the obstruction causes a rapid return of the blood to normal and allows the animal to recover. A

similar amount of fluid given as 2 per cent glucose does not alter the course of the toxemia after release of the obstruction.

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THE BLOOD CHLORIDES IN PROTEOSE INTOXICATION.

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The characteristic chemical changes in the blood after upper gastro-intestinal tract obstruction are: a fall in chlorides, a rise in the CO_2 combining power, and an increase in the non-protein nitrogen and urea nitrogen (1). These changes have been interpreted by some as due solely to the increased loss of water and salts through vomiting and increased excretion into the intestinal tract with consequent dehydration and kidney retention. Others think there is a primary chemical intoxication which is at least one factor in the characteristic blood changes. Toxic substances may come from the intestinal mucosa, from the activity of bacteria within the lumen of the intestine, or possibly be formed in the process of protein disintegration throughout the body.

Whipple and his coworkers (2) have emphasized especially the rôle of proteoses in the intoxication. These may be isolated from the contents of the obstructed gut and arise, they think, from the intestinal mucosa. It has been shown that the symptoms characteristic of intestinal obstruction may be reproduced by the intravenous injection of the proteoses. Animals so injected show a rise in the non-protein nitrogen and urea nitrogen of the blood with an increased nitrogen excretion.

In certain other chemical intoxications such as in burns (3), x-ray intoxication (4), and lobar pneumonia (5), the chloride metabolism is similar to that observed in intestinal obstruction. These facts together with other observations in intestinal obstruction suggest that the chloride loss through vomiting and increased secretion into the intestinal tract may not entirely account for the changes in blood chloride. In seeking other possible causes for the loss in chlorides we have studied the blood chemistry of dogs with different types of proteose intoxication with special reference to the chlorides.

TABLE I.

Chemical Findings after Injection of Proteose from Intestinal Content of Dogs with Obstruction at Different Levels.

Dog No.	Hrs. after injection	Blood (amount per 100 cc.)				Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides as NaCl	CO_2 combining power	
		mg.	*mg.*	*mg.*	*vol. per cent*	
3-26	0	25.4	10.7	480	30.5	Intestinal content from dog with obstruction of jejunum
	2	31.6	17.5	460	18.3	Vomiting and diarrhea
	4	38.5	16.1	410	14.5	Died during night
3-50	0	25.0	12.6	480	32.4	Intestinal content from dog with obstruction of jejunum
	2	34.1	19.6	490	21.9	Vomiting and diarrhea
	4	42.2	23.8	460	17.2	Died during night
3-49	0	28.0	16.8	460	34.3	Intestinal content from dog with pyloric obstruction
	2	36.6	17.5	500	21.9	
	4	45.6	20.3	490	21.9	Vomiting and diarrhea
	24	38.9	18.2	480	26.6	Recovered
3-48	0	28.2	15.4	490	22.8	Intestinal content from dog with pyloric obstruction
	2	55.5	21.0	480	11.0	
	4	53.5	25.9	500	7.2	
	24	79.0	43.3	450	13.4	Died
3-32	0	25.0	12.6	470	39.3	Intestinal content from dog with cardiac obstruction
	2	37.0	22.4	470	5.0	Died
3-45	0	29.7	14.0	460	33.4	Intestinal content of dog with cardiac obstruction
	2	40.0	17.5	490	32.4	
	4	37.5	19.6	490	24.7	
	24	40.0	18.3	470	24.7	No symptoms

TABLE II.

Blood Chemical Findings after the Intravenous Injection of Witte's Peptone.

Dog No.	Hrs. after injection	Blood (amount per 100 cc.)				Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	
		mg.	*mg.*	*mg.*	*vol. per cent*	
6-04	0	36.1	25.7	470	30.5	50 cc. 5 per cent peptone intravenously
(wt.	¼	36.6	24.8	420	10.7	
12 kg.)	6	66.3	32.7	420	10.7	Died following night
6-05	0	33.3	16.8	540	32.4	50 cc. 5 per cent peptone intravenously
(wt.	¼	37.5	18.2	500		
15 kg.)	3	48.2	23.4	540	12.9	Died
6-18	0	30.0	14.5	520	30.5	50 cc. 5 per cent peptone intravenously
(wt.	¼	43.6	22.4	480	9.2	
6.7 kg).	3	50.0	29.0	510	10.7	Died
6-02	0	38.5	17.3	500	40.9	50 cc. 5 per cent peptone intravenously
(wt.	¼	38.9	17.3	520	32.4	
8 kg.).	2	41.6	23.35	460		
	4	37.0	24.3	480	30.5	
	24	52.6	22.0	360	34.3	50 cc. 5 per cent peptone intravenously
	27	47.6	29.0	470	30.5	
	30	37.0	22.0	450	30.5	
	48	52.8	38.8	490	34.3	Recovered
6-73	0	30.0	7.0	490	37.2	50 cc. 5 per cent peptone intravenously
(wt.	20	20.3	4.7	470	32.4	50 " 5 " " " "
10.7 kg.).	44	26.3	4.7	470		50 " 5 " " " "
	68	24.2	8.4	440	36.2	50 " 5 " " " "
	92	25.0	9.3	380	33.4	50 " 5 " " " "
	116	28.7	6.5	480	38.1	50 " 5 " " " "
	140		45.8	440	39.0	50 " 5 " " " "
	146					Died

Method.

Proteose was prepared from the intestinal contents by the method described by Ellis (6) and dissolved in a small amount of distilled water for injection. All injections were made intravenously. The animals were bled from the jugular vein before injection and at intervals thereafter as indicated in the tables.

TABLE III.

Chemical Findings after Intravenous Injection of Proteose from Intestine of Dogs with Obstruction but without Toxemia (Treated with Sodium Chloride).

Dog No.	Hrs. after injection	Blood (amount per 100 cc.)				Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	
		mg.	*mg.*	*mg.*	*vol. per cent*	
3-23	0	28.5	12.6	410	25.6	
	2	33.3	19.6	410	12.6	
	4	36.1	17.5	400	18.0	No symptoms
3-27	0	31.2	14.0	470	36.2	
	2	40.0	18.9	460	10.1	Vomiting and diarrhea
	4	62.5	30.8	460	10.1	Died soon after
3-41	0	34.9	15.4	500	30.5	
	2	36.1	18.2	510	17.2	
	4	38.5	19.6	500	19.5	Vomiting
	24	30.3	13.1	500	27.5	Recovered
3-51	0	25.2	10.5	470	22.8	
	2	27.2	13.3	500	20.0	Vomiting and diarrhea
	4	25.4	11.9	500	14.3	
	24	26.1	11.9	490	20.0	Recovered

In the liver experiment liver was obtained with sterile precautions from one animal and placed free in the abdomen of another. All operations were done under ether anesthesia, with aseptic technique. The non-protein nitrogen was determined by the method of Folin and Wu (7), the CO_2 combining power by the method of Van Slyke and Cullen (8), the urea nitrogen by the Van Slyke and Cullen (9) modification of the Marshall method, and the chlorides on the tungstic acid filtrate in the manner described by Gettler (10).

EXPERIMENTAL OBSERVATIONS.

A large number of experiments were made. Typical results are shown in the tables. In Table I are recorded the blood chemical findings in animals injected with the proteose from other animals with obstruction at different levels. All show some increase in the urea nitrogen and non-protein nitrogen with a fall in the CO_2 combining power. Four of the six animals died within 24 hours. Most of them had vomiting and diarrhea. The chlorides show very little change.

TABLE IV.

Chemical Findings in Blood of Dog with Free Liver in Abdomen.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	
		mg.	*mg.*	*mg.*	*vol. per cent*	
5-25	0	24.8	8.4	490	40.9	7.5 gm. of liver placed in abdomen
	1	29.1	9.1	490	40.0	
	2	32.6	12.2	480	34.3	
	3	27.0	8.4	490	32.4	
	4	27.3	7.0	490	32.4	
	5	34.5	10.5	570	30.5	Died, autopsy negative
5-33	0	41.6	18.9	500	36.2	12.5 gm. of liver placed in abdomen
	1	44.4	15.4	460	36.2	
	2	54.6	19.6	460	40.0	
	3	17.7	77.1	510	29.6	
	4	24.3	112.8	500	27.5	Died. No peritonitis. Small amount hemorrhagic fluid in abdomen

As a control on these experiments several animals were injected with Witte's peptone which consists largely of proteoses. These showed changes similar to those observed after injection of proteose from animals with experimental obstruction. The chlorides again showed no significant changes.

Animals dying of gastrointestinal tract toxemia usually show at autopsy a markedly distended gall bladder. Several dogs were injected with the proteose recovered from the bile of animals with

obstruction at different levels. One animal 24 hours after injection, showed a non-protein nitrogen of 124 mg. without significant changes in the chlorides. Two control animals and several others injected with material from obstructed animals showed very little change.

It has been shown that injections of sodium chloride solution will prolong the life of animals with high intestinal obstruction and prevent the development of a toxemia. Four dogs were injected with the proteose recovered from the obstructed intestine of dogs in which the development of a toxemia was prevented by the injection of salt solution. The results are shown in Table III. One animal showed a marked increase in non-protein nitrogen, two showed clinical evidence of intoxication with blood changes, and one showed no symptoms.

Davidson and Mason (11) found that a proteose intoxication could be easily produced in animals by placing liver free in the abdomen. The rise in non-protein nitrogen which takes place in such animals is due to the absorption of autolyzed protein. The blood chlorides were studied in two such animals. One showed very little change in the blood chemistry. One had a very great rise in urea nitrogen and non-protein nitrogen without significant change in CO_2 combining power or chlorides.

DISCUSSION.

The chemical changes in the blood produced by the injection of proteose were not so marked as those found by Whipple. This may have been due to the fact that a purified proteose was employed. In purifying the proteoses from the intestinal contents, either the proteoses became less toxic or some toxic substance is removed. In this series however, four of six animals injected died.

There is no definite change in the chlorides even with a marked rise in non-protein nitrogen. Likewise with a marked intoxication resulting from the injection of Witte's peptone, and in dogs with autolyzing liver within the abdomen, there is no significant change in the chlorides.

These findings would indicate that proteose intoxication is not concerned in the characteristic fall in blood chlorides observed after intestinal and pyloric obstruction.

SUMMARY.

Dogs injected with proteose recovered from the intestinal contents of animals with obstruction at different levels show no significant changes in the blood chlorides even with a fatal intoxication.

After the intravenous injection of lethal and sublethal doses of Witte's peptone there is little change in the chlorides.

Autolyzing liver in the abdominal cavity produces no change in the blood chlorides even with a very great increase in the urea and non-protein nitrogen.

Proteose intoxication is probably not a factor in the characteristic fall in chlorides seen after intestinal and pyloric obstruction.

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STUDIES ON A PARATYPHOID INFECTION IN GUINEA PIGS.

V. The Incidence of Carriers during the Endemic Stage.

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The course of two Salmonella infections appearing spontaneously in a guinea pig population has been discussed in previous papers (1, 2). As noted earlier, the guinea pigs comprising the particular population are divided into two groups, breeders and weaned young or stock. Animals employed in experimental work are withdrawn from the latter group. From time to time *B. paratyphi* has been isolated from the spleen of stock guinea pigs injected with other bacteria. It seemed probable that the guinea pigs were carrying *B. paratyphi* in the spleen as a result of earlier exposure to infection.

A routine bacteriological examination of all guinea pigs that died had been carried out for several years. Positive cultures were rarely obtained from the spleen with unweaned guinea pigs in the absence of gross lesions. However, no definite study of carriers within the population at large had been attempted. Consequently a comprehensive examination of breeders and young guinea pigs, just prior to weaning, was undertaken. In addition a new breeding group composed of selected sows and boars was brought together and isolated from the main population. The selected group was kept under observation for evidence of reinfection.

Before considering the work on carriers a more detailed statement concerning the extent of the two infections may be pertinent. The specific percentage mortality for the population (including the selected breeders) during that year is given in Table I. The usual monthly fluctuations occurred. The rates for the second infection, introduced into the population a year earlier, were in general higher than those for the first form.

When the examination for carriers was begun the breeding stock was composed of some 209 animals. These were maintained in a separate animal unit, distributed in large metal cages containing 4 or 5 sows and 1 boar. The group was a heterogeneous one comprising animals from 1 to 5 years of age. A few of the number had passed through the active stage of the initial outbreak of paratyphoid in 1924. The majority of breeders, however, were younger animals added shortly before or at intervals after the appearance of the second infection during the summer of 1926. Generally the individuals in any one cage were of approximately the same age.

Cages containing a full quota of guinea pigs were selected and brought to the laboratory for examination. It may be said that pregnant sows are removed to

TABLE I.

Population, Total Deaths, Deaths from Paratyphoid, and Percentage Mortality from Paratyphoid from September, 1927, through June, 1928.

Month	Population	Total deaths	Deaths from Paratyphoid Type I	Mortality Type I	Deaths from Paratyphoid Type II	Mortality Type II
				per cent		*per cent*
September	513	11	1	0.19	6	1.16
October	561	17	3	0.53	11	1.96
November	668	23	1	0.14	5	0.74
December	573	27	2	0.34	3	0.52
January	661	60	3	0.45	13	1.96
February	631	39	2	0.31	6	0.95
March	595	62	0	0.00	1	0.16
April	497	73	1	0.20	1	0.20
May	493	43	2	0.40	1	0.20
June	483	75	1	0.20	3	0.62

individual units just prior to parturition. In all, 105 animals were transferred, representing 20 cages. The entire work extended over a period of 5 months. Two successive fecal cultures were made from each guinea pig and the agglutinin content of the blood serum determined for the two types of *B. paratyphi*.

General Methods.

The following routine procedures were carried out.

The guinea pigs were first bled. In most instances sufficient blood was obtained from incising an ear vein after shaving and the application of xylol. 13 of the total number were refractory and heart puncture under ether was resorted to, with no fatalities. Serum from the coagulated samples was used in

testing. The antigens were fresh, standardized, saline suspensions of the two Salmonella types. The final dilution series in a total volume of 1 cc. ranged from 1:20 through 1:1280. The tubes were incubated at 37°C. for 3 hours and were read after overnight refrigeration. In Tables III and IV the results are expressed in terms of the limiting dilution, the highest dilution showing any macroscopic evidence of agglutination.

After bleeding, the guinea pigs were transferred to separate cages with clean bedding. The next morning a fecal sample was collected. The bedding was changed and on the following morning a second sample collected. The fresh feces, at least two pellets, were emulsified in 5 cc. of plain broth and incubated for 5 to 6 hours at 37°C. Two large loops were then transferred to a tube of malachite green-lead acetate broth.

Difficulty had been experienced with this medium in former work. Hydrogen sulfide in amounts detectable by lead acetate was produced only when fermented bouillon was employed. The peptone used was apparently deficient in the particular sulfur compound utilized for the production of hydrogen sulfide. With the fermented bouillon some available sulfur compound was evidently produced during growth of the fermenting bacteria. It may be noted that *B. coli* is added to the meat infusion and allowed to grow at incubator temperature. After steaming and filtering, peptone is added as usual. Tilley (3) recommended the addition of sodium thiosulfate to media employed for hydrogen sulfide production. This compound was substituted for fermented broth with excellent results. The medium as finally employed was beef infusion broth containing 1 per cent peptone, of an initial pH of 7.6, and tubed in 5 cc. amounts. Immediately before use there was added 0.05 cc. of a 5 per cent aqueous solution of sodium thiosulfate, 0.25 cc. of a 1 per cent solution of lead acetate, and 0.15 cc. of a 0.2 per cent aqueous malachite green solution. The addition of the lead acetate causes a bulky precipitate which settles rapidly. In this medium *B. paratyphi* and other members of the Salmonella group produce a uniformly turbid growth. The precipitate blackens during the first 24 hours and there is a distinct odor of hydrogen sulfide. Motility is retained but is less active than in plain bouillon. Other intestinal bacteria are generally restrained through 48 hours at 37°C. *B. proteus*, however, may develop with the production of hydrogen sulfide. Occasionally there is growth of unidentified bacteria with no change in color of the precipitate.

The tube of the above medium inoculated from the fecal broth culture was incubated through 48 hours at 37°C. In the absence of growth or in the presence of growth with no motility and no change in color of the precipitate the tube was discarded. In the presence of growth, motility, and a black precipitate a loopful was streaked on the surface of a Petri plate containing lead acetate-thiosulfate agar. A thin film of the same medium was layered over the inoculated surface. Under this semianaerobic condition *B. paratyphi* produces large dark brown colonies. In the presence of such colonies transfers were made from several to lactose and saccharose fermentation tubes. In the absence of acid and gas the

cultures were confirmed by agglutination with the two type antiserums. With each lot of fecal cultures a medium control inoculated from a young broth culture of *B. paratyphi* was included. The above procedure was followed in all the subsequent work.

The Incidence of Carriers among the Breeders as Determined by Cultural and Serological Examination.

Of the 105 guinea pigs examined 69 were added to the breeding stock after the appearance of the second infection. Only 4 were survivors of the initial outbreaks in 1924. An agglutination test with the two types of *B. paratyphi* as antigens was made on serum from each animal. Eight samples failed to agglutinate either type in the lowest dilution

TABLE II.

Number of Samples Showing Agglutination of One or Both Types of B. paratyphi at Each Dilution Interval.

Serum dilution	1:20			1:40			1:80			1:160			1:320			1:640			1:1280		
Type of bacillus	I	II	Both	I	II	Both	I	II	Both	I	II	Both	I	II	Both	I	II	Both	I	II	Both
Number of samples	0	11	7	3	5	5	1	22	1	1	15	1	4	12	2	2	5	0	0	0	0

employed. The remaining samples agglutinated one or both of the antigens in dilutions ranging from 1:20 through 1:640. With 66 serums of the latter group the dilution was 1:80 or higher. A detailed classification of the samples as to limiting dilution for the major agglutinin is given in Table II. Only 5 of the 24 males included in the group examined showed an agglutinin titer higher than 1:80 as compared with 45 high reactors among the 81 females. Two serums which agglutinated one type through a dilution of 1:160 failed to agglutinate the other type in 1:20. The majority of serums, however, agglutinated both types generally with a difference of one or two dilutions between them. With 70 samples the major agglutinin (highest limiting dilution) was for Type II antigen as compared with 11 for Type I.

B. paratyphi was isolated from the feces of 3 guinea pigs of the group examined.

One was an old sow added to the breeding stock in the fall of 1924 shortly after the termination of the active stage of the initial infection. Type II *B. paratyphi* was cultivated from the feces on 2 successive days. The blood serum agglutinated Type I antigen through 1:160, Type II antigen through 1:320. The animal was kept under observation for 3 weeks and then killed. The uterus contained 3 half-term fetuses. The surface of the spleen was scarred and pitted. Other organs were normal. Cultures from the spleen, placental tissue, and cecal feces were all negative.

The other positive cases were younger sows added to the breeding stock in December, 1926, and July, 1927, respectively, before and after the appearance of the second infection. The former was an active case. *B. paratyphi*, Type II, was isolated from the first fecal culture. The next morning the animal was weak with labored breathing and was killed. The blood serum agglutinated Type I antigen through 1:160, Type II through 1:640. At autopsy a peritonitis was encountered. A heavy exudative membrane was reflected over the surface of the spleen and portions of the liver. The abdominal cavity contained a quantity of turbid fluid. Type II *B. paratyphi* was isolated from the spleen, liver, and cecal feces.

The second of the younger animals was a carrier. A Type II organism was cultivated from the feces on successive days. The blood serum agglutinated Type I antigen through 1:40, Type II through 1:80. After 2 weeks the animal was killed and autopsied. The uterus contained 2 nearly full-term fetuses. The spleen was atrophic and extensively roughened and pitted. Cultures from the spleen, placental tissue, cecal feces, and fetal spleens were all negative.

The Incidence of B. paratyphi among a Group of Selected Breeders.

All guinea pigs from the tested group which showed consecutively negative fecal cultures and with an agglutinin titer of 1:80 or lower were segregated as a nucleus for a new breeding stock. The carriers were killed, as noted. The guinea pigs which showed high agglutinin content were returned, in most instances, to their original unit and isolated in special cages. A few were killed and autopsied. The selected animals were isolated in a separate unit in groups numbering up to 5 sows and 1 boar. The two units were maintained independently.

During the 5 months occupied in testing the entire group additional fecal cultures were taken at intervals from the segregated individuals. At first, mass cultures were made from the breeding cages. During one examination which comprised eight cages a culture of Type I *B. paratyphi* was obtained from the feces of a single cage. The guinea pigs were immediately transferred to individual

pens and cultures made on 2 successive days. The positive culture was traced to a single sow from which the same type of organism was isolated. The animal was removed and later killed. At autopsy no gross lesions were encountered and cultures from the spleen, uterus, and cecum were all negative. The blood serum agglutinated Type I antigen through 1:160, Type II through 1:20. Fecal cultures from the other inmates of the cage were all negative.

For the third fecal examination the guinea pigs were isolated in individual cages and a single culture made. The group comprised 46 adult animals distributed in ten cages. In addition there were 22 younger breeders representing the second generation of the selected population. The entire lot of fecal cultures was negative.

The segregated group was maintained in the same manner as the main breeding stock. Pregnant sows were removed to individual cages shortly before parturition. Young guinea pigs were kept with their dam for 2 to 3 weeks and then weaned. The sow was returned to her original breeding cage. The young were separated as to sex and placed in large open pens. A few of the weaned young were used for experimental work. 9 of these were carefully examined subsequent to infection with other bacteria. Cultures from the entire spleen were negative throughout. The majority of them were allowed to mature for breeding purposes. Before admission to the breeding stock they were isolated in individual cages and fecal cultures made.

The selected population was kept under observation and all animals that died were autopsied and cultured. Through June, 1928, a total of 111 guinea pigs was examined. The majority of them were still-born or unweaned young. *B. paratyphi* was isolated from the spleen on four occasions.

The first culture was obtained in February from a female stock guinea pig. At autopsy a large amount of turbid fluid was found in the pleural and peritoneal cavities. The spleen was enlarged and coated with a heavy, tenacious exudate which was likewise reflected over portions of the liver. The cervical and peritoneal lymph nodes were enlarged and congested. Type I *B. paratyphi* was isolated from the spleen, gall bladder, pleural fluid, and cecum.

The second culture was isolated from an unweaned male guinea pig whose dam had died on the previous day. At autopsy the lymphoid tissue of the cecum was congested and a culture of Type II *B. paratyphi* was secured from the spleen. The dam showed no evidence of infection. The spleen failed to show *B. paratyphi*.

The third and the fourth cultures were isolated in June, in both cases from sows. At autopsy, the first animal showed an enlarged and congested spleen. Both spleen and liver were coated with a tenacious exudate. The gall bladder contained purulent fluid. There was an inflammation of the small intestine. The abdominal cavity contained a mucoid fluid. Type II *B. paratyphi* was isolated from the spleen. The second sow was carrying 4 nearly full-term fetuses. No gross manifestations of infection were encountered. Type I *B. paratyphi* was cultivated from the spleen.

The Incidence of B. paratyphi among Unweaned Young of the Main Population.

A less extensive study was made of the incidence of carriers among the unweaned young of the main population.

Monthly examination of 6 guinea pigs from as many litters, chosen at random, was carried out during the period between September, 1927, and February, 1928. The animals varied in age from 2 to 3 weeks. They were brought to the laboratory, bled from the heart under ether, and killed. Postmortem examination was made and cultures taken from the spleen, gall bladder, small intestine at a Peyer's patch, and cecal feces.

During October, 2 fecal carriers and 1 active case of paratyphoid were encountered. All 3 were females, 3 weeks old. Type II *B. paratyphi* was isolated from the cecum of the 2 carriers. The abdominal organs showed no gross changes. Cultures from the spleen, gall bladder, and small intestine were negative in each instance. The active case showed typical gross changes in the abdominal viscera. The spleen was enlarged, congested, and showed a single nodular focus. The liver showed a few small foci. There were numerous exudative plaques on the surface of the cecum with foci in the lymphoid tissue at the head. Type II *B. paratyphi* was obtained from the spleen, small intestine, and cecal feces. During the same month 3 active cases occurred among the young of a second sow from the same breeding cage. In February and March fatal cases were noted among the young of 2 sows originally caged with the dam of the first carrier, mentioned above. All of the unweaned guinea pigs examined during the remaining months were normal with negative cultures throughout.

Agglutination tests were made on blood samples from all the unweaned guinea pigs. The procedure previously outlined was followed except that the dilution ranged from 1:10 through 1:640. The results expressed in terms of the limiting dilution showing agglutination are given in Table III. Seven samples failed to agglutinate either type of antigen in the lowest dilution. Four samples agglutinated both types through the same limiting dilutions. The remainder agglutinated both types of antigen but the limits of reaction varied by one or more dilutions. With eighteen samples of this number the major reaction was against Type II, while with seven the major reaction was against Type I. Classified according to sex it may be noted that serums from 2 of the 12 males and 5 of the 24 females were negative. Serum from the first of the fecal carriers agglutinated Type I through 1:20, Type II through 1:80; that from the second agglutinated Type I through 1:20, Type II through 1:40. There was no agglutination against either type with serum from the active case.

Agglutination tests were also made on serum from the dams of 3 unweaned guinea pigs and from the remaining young of their litters. The findings, which suggest maternal transmission of antibodies to the suckling young, are given in

TABLE III.

Limiting Agglutination with Serum from Unweaned Guinea Pigs.

Month	Type of bacillus	Limiting dilution of serums					
Guinea pig No.....		1	2	3	4	5	6
September	I	40	20	40	80	20	10
	II	80	40	40	40	80	20
October	I	20	20	20	20	—	—
	II	20	80	40	80	—	—
November	I	20	40	20	40	20	20
	II	80	20	80	10	80	160
December	I	80	40	80	10	—	—
	II	20	80	40	20	—	10
January	I	—	—	10	—	10	10
	II	—	—	20	—	20	20
February	I	20	20	—	10	80	20
	II	80	—	—	10	20	20

TABLE IV.

Agglutinin Titer of Serum from Sows and Their Unweaned Young.

Class of guinea pig	Limiting dilution	
	Type I	Type II
1st young.....	10	10
Sow.....	80	20
2nd young.....	20	10
3rd "	20	10
1st "	80	20
Sow.....	160	20
2nd young.....	40	10
3rd "	40	10
1st "	20	80
Sow.....	40	320
2nd young.....	20	80
3rd "	20	80

Table IV. It may be said that all the animals were later killed with negative pathological and bacteriological findings throughout. There was an interval of 4 days between the examination of the first young and the other guinea pigs of the group.

DISCUSSION.

The examination of breeders described in the foregoing pages was begun in October, 1927. 13 months earlier the second type of Salmonella infection had appeared spontaneously in the population. There had been a slow but general spread of the second bacterium throughout the breeding group. Specific deaths occurred among the sows or the unweaned young of sows from 32 of the 48 cages. The Type I infection had long since become endemic, with the mortality rate in general on a low level.

The serological findings appear to reflect the distribution of the two Salmonella types. 92 per cent of the 105 serums tested showed well defined agglutination against one or both types of *B. paratyphi* in dilutions ranging from 1:20 through 1:640. With 86 per cent of the reactors the higher agglutination was against the second, more recent type of organism. With 13 per cent the higher agglutination was against the initial form. In each case the majority of serums agglutinated both types although one in lower dilution. The physical form of the sediment was not sufficiently defined to permit a distinction between common agglutinins for the one type and specific agglutinins for the other.

It was assumed that the high agglutinin content of individual serums was referable to a multiplication of one or both strains in the system of the guinea pig. Postmortem cultures from a small series of high reacting guinea pigs failed, however, to demonstrate the specific organism in the spleen, liver, or section of the small intestine. These observations suggest that a high agglutinin titer may be maintained in the absence of a persistent localization of *B. paratyphi* in the more usual sites. The bacteriological examination, however, was not exhaustive. The entire spleen was cultured but examination of the liver and small intestine was necessarily limited. Moreover, the possible localization of the specific bacterium in regional lymph nodes is not ruled out. Temporary colonization of *B. paratyphi* in one or another of these sites might well impart the stimulus leading to antibody formation.

The serological findings with the unweaned guinea pigs roughly parallel those with the breeders. The total number of reactors while high was less than with the mature animals, 80 per cent as compared with 92 per cent. The young guinea pigs also showed a greater number of high reactors against the second type of *B. paratyphi* than against the first. The difference was not as marked, however, as with the older animals. The agglutinin titer in general was on a lower level than that of the breeders. The highest limiting dilution recorded was 1:160, with the majority of serums well below that figure. A number of serums from the breeders agglutinated through 1:640 with a fair proportion showing reaction between 1:80 and 1:320. There was a suggestion that the agglutinin content of the young was referable, in part, to placental transmission or to lactation. Sublethal infection at birth with immunization was not excluded as a possible cause of the presence of agglutinins in the group at large.

By way of comparison it may be of interest to note the serological findings reported by Amoss (4) on the survivors from an artificially induced paratyphoid epidemic in mice. A population of 300 mice comprising susceptible individuals in contact with a previously infected group was set up. During the epidemic wave which followed, 69 per cent of the population died. He tested the serum from 56 of the survivors for agglutinin against the causal organism, Mouse Typhoid II. The serum from 37 or 66 per cent partially agglutinated the organism in dilutions ranging from 1:20 to 1:160. The serum from 27 showed no agglutination in 1:20.

The cultural tests in the present work indicated a low incidence of fecal carriage among the breeders. *B. paratyphi* was isolated from the feces of only 3 sows, 1 of them an active case. A group of 50 guinea pigs selected from the total number examined was again cultured on three occasions with the detection of a single additional carrier. While the results cannot be regarded as strictly quantitative it is believed that the figure cited is a fair index of the extent of specific fecal excretion in the breeding group as a whole. Some of the animals examined were selected from cages in which fatal cases of paratyphoid had previously occurred. Conditions were favorable for the ingestion of the specific bacteria with food soiled by fecal excretions. In the face of such conditions the small number of fecal carriers actually detected

seems to bear out the suggestion made in a previous paper that specific fecal excretion with adult guinea pigs is of relatively short duration (5).

Examination of the unweaned young showed that fecal carriage of *B. paratyphi* may occur with nursing guinea pigs in the absence of generalized infection. *B. paratyphi* was isolated from the feces of 2 guinea pigs which showed no manifestations of disease. The group tested was too small to indicate the actual extent of such carriage. The young guinea pigs after weaning are transferred to the general stock for laboratory use. The presence of fecal carriers among them may account for the subsequent occurrence of sporadic cases or carriers. It is known that *B. paratyphi* may localize in the spleen or liver with no indication of multiplication or injury. It is possible that the introduction of other bacteria into such guinea pigs may be followed by multiplication of the paratyphoid types with active disease.

Both types of *B. paratyphi* appeared among the guinea pigs of the segregated group. The selected population which included breeders and later unweaned young and stock was maintained in a separate unit. There was no communication with the main supply of guinea pigs. Chance contact with field animals was practically impossible. Introduction of the specific bacteria with the food was not entirely ruled out. It seemed more probable, however, that the initial selection was not sufficiently rigid and that a number of spleen or fecal carriers were admitted to the new breeding stock. To date (June, 1928) there has been no evidence of extensive dissemination of the bacteria throughout the population.

SUMMARY.

A group of 105 breeders and 36 unweaned guinea pigs was tested to determine the extent of specific fecal excretion and the proportion of serum reactors in a population naturally infected with two types of *B. paratyphi*. The second, more recent type of organism was isolated from the feces of 3 breeders and 3 young. No carriers of the first type were detected. 86 per cent of the breeders and 72 per cent of the unweaned guinea pigs agglutinated the second type of *B. paratyphi*

in dilutions ranging from 1:10 through 1:640. 13 per cent of the breeders and 28 per cent of the unweaned young agglutinated the initial type. There was a cross or double agglutination in most instances. The serological findings roughly reflected the distribution of the two types as indicated by the mortality rate of the population at large.

50 breeders selected on the basis of agglutination and fecal examination and therefore supposedly free from infection were segregated and kept under close observation. Both types of *B. paratyphi* subsequently appeared in the group.

During this time carriers were discovered by others in the Department among the stock guinea pigs used for other experiments in that cultures of the entire spleen were positive in perhaps 5 to 10 per cent of the guinea pigs so used.

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STUDIES IN THE BIOLOGY OF METALS.

VII. The Influence of Lead on the Development of the Chick Embryo.

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(Received for publication, July 3, 1928.)

INTRODUCTION.

A knowledge of the fundamental biological reactions to metals is a prerequisite to their intelligent use in the fight against disease. It has long been known that lead retards growth, but the studies so far made have dealt merely with the gross response (1–5). They are thus inadequate save as confirmations of a predictable reaction. They tell nothing of the differential response, or whether it is growth by increase in cell size or growth by increase in cell number which is affected. This distinction is of importance if the use of lead or any other metal as an inhibitant of pathological growth is to have a rational basis.

As a first step in the analysis of this phase of metal activity a series of studies was made of the influence of lead on cell growth and mitosis in root tips. The results are reported in preceding papers. Briefly, it has been established by this work that lead retards growth because it inhibits mitosis or cell proliferation, not because it inhibits cell growth in size (6). It does this because certain chemical properties of the nucleus, particularly the nucleus in mitosis, make possible the concentration of lead therein (7, 8), thus precipitating out of action substances essential for cell division (9).

While there is every reason to suppose from both chemical and morphological evidence, that the phenomenon of mitosis is fundamentally the same in plants and animals, and hence that the basis of growth inhibition by lead would be the same in each, yet supposition is not proof. For this reason a repetition of the plant experiments

was begun on the animal. The chick embryo was chosen because material is at all times available, because controlled cultivation is possible, and because its developmental stages are well known and accurately determinable by somite count.

Procedure.

A series of embryos was subjected to lead during growth as follows: Clean, unincubated eggs were lightly brushed with 80 per cent alcohol. They were allowed to stand under sterile conditions for 10 or 15 minutes until the rotation of the yolk had brought the blastoderm to the top. The egg was then lightly and quickly flamed with an alcohol lamp and placed on an appropriately designed sterile holder. The top surface was dabbed with alcohol and a window cut in the shell with a safety-razor blade. The blastoderm now being in view, 0.4 to 0.5 cc. of yolk was removed and replaced with the same volume of $Pb(NO_3)_2$ solution containing 0.05 per cent Pb ion. This was done with a micro syringe. Care was taken to avoid injury through stretching of the yolk sac during the puncture, or through making the injection too rapidly or too near the blastoderm. The lead was injected under the blastoderm through the same hole that was used for the withdrawal of the yolk. The membrane and shell were then replaced and the edges sealed with paraffin. Precautions for the maintenence of sterility were rigorously taken. The egg was then placed in the incubator with the window *not* on top. By this detail the embryo is brought to the top of the egg out of the zone of tension caused by the cut in the external membrane. It thus escapes mechanical distortion which much experience has shown to be the cause of abnormal development and high mortality.

After incubation for 18 to 72 hours the eggs were taken from the incubator. The living embryos were gently cut from the yolk sac, washed free from yolk in warm saline with brush and pipette, dehydrated in increasing concentrations of alcohol, cleared in xylol, stained with neutral red and Janus green, and mounted in balsam. In this way a series of leaded embryos from the head fold to the 30 somite stage was obtained.

For comparison three sets of controls were prepared. The first consisted of normal embryos, the second of embryos from eggs in which the window alone had been cut, and the third of embryos subjected to 0.8 per cent NaCl instead of lead. The reaction to lead was determined by comparing the tests with the controls of equal somite number. This, rather than the time of incubation, is the accepted index of degree of development in the early stages.

Fresh, clean, fertile eggs from the White Leghorn and Barred Plymouth Rock breeding stock of the Pratt Experiment Farm were used.

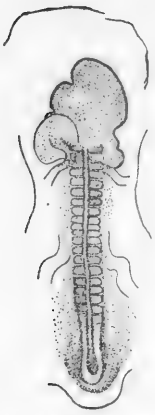

FIG. 1 *a*. Leaded embryo (1-4′) of 20 somites.

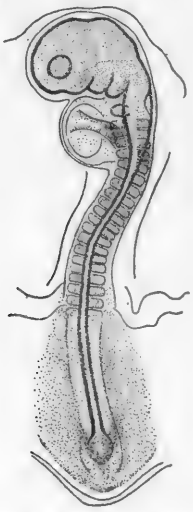

FIG. 1 *b*. Normal embryo (N-8) of 20 somites.

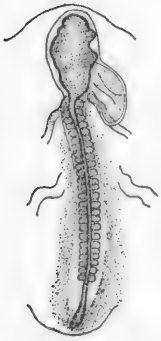

FIG. 2 *a*. Leaded embryo (L-9) of 26 somites.

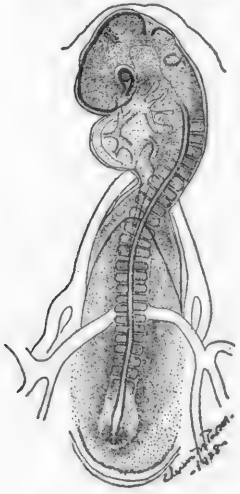

FIG. 2 *b*. Normal embryo (N-12) of 26 somites.

Drawings of normal chick embryos and of embryos subjected to lead during development.

RESULTS.

The most striking response to the presence of lead is the retardation of the differential development of the head end of the embryo. This is exhibited in the youngest head fold stage as well as in the one of 30 somites or more. The phenomenon is shown in Figs. 1 *a*, 1 *b*, 2 *a*, and 2 *b* where leaded embryos of 20 and 26 somites are compared with their normal controls. The absence of progressive differentiation is marked in these two characteristic specimens. In less affected embryos, even though growth has proceeded, the retardation of development of the optic anlagen is clearly evident.

This selective type of inhibition does not follow the disturbance caused by cutting the window, nor when there is added to this the removal of 0.5 cc. of yolk and its replacement with saline solution. In the latter case, indeed, the impression is given of a tendency to an overdevelopment of the head region. This was not confirmed by detailed study since our interest was primarily in the lead reaction.

The somites are also the seat of a differential retardation. When the width and length of these active centers of cell proliferation are measured in embryos which develop in contact with lead, it is found that they are not only absolutely, but also relatively smaller than the same-placed structures in the controls of equal somite count. This is determined by the quotient of embryo dimension into somite dimension. The data over a wide range of development are given in Table I.

The consistency of the difference at the several stages establishes the reaction as definite. Its significance is determined by the fact that it appears neither in the window controls nor in the saline controls. In other words, lead produces a specific differential retardation of somite growth.

A third result is that the embryos which grow in contact with lead are smaller than their controls of equal somite number. This retardation of gross growth is of course what was to have been expected. It is essentially specific in that it does not occur when the eggs are only opened, or opened and injected with saline.

A distinction needs to be made here between embryo size as based on somite count, and size as based on duration of incubation. The

TABLE I.

Somite and Embryo Dimensions in Lead-Treated Chicks and Their Controls.

Embryo No.	Somites measured	Somite		Embryo		S/E	
		Width 0.01 mm.	Length 0.01 mm.	Width 0.01 mm.	Length 0.01 mm.	Width	Length
						per cent	*per cent*
				15 somites			
Pb-L3	13	8.1	8.2	109.6	260.3	7.4	3.2
Cont.-N7c	13	13.2	10.3	123.3	287.7	10.7	3.6
				21 somites			
Pb-L4	14						
Pb-L4′	15 av.	8.9	8.6	123.3	311.0	7.2	2.8
Pb-L5	12						
Cont.-N8	14						
Cont.-N9	10 av.	12.6	12.1	141.1	397.3	8.8	3.0
Cont.-N10	10						
Cont.-S8	14	13.4	11.4	150.7	383.6	8.9	3.0
Cont.-W14′	10						
Cont.-W15	13 av.	15.9	11.9	178.1	411.0	8.9	2.9
				23 somites			
Pb-L7	10						
Pb-L7′	14 av.	11.4	10.7	143.9	404.2	7.9	2.6
Cont.-N11	12						
Cont.-N11′	14 av.	15.3	15.2	171.3	534.3	9.0	2.8
Cont.-S9	13						
Cont.-S9′	15 av.	12.1	12.3	130.2	417.9	9.3	3.0
Cont.-W16′	11						
Cont.-W17	12 av.	15.1	13.7	164.4	438.4	9.2	3.1
				26 somites			
Pb-L8	10						
Pb-L9	20 av.	12.9	10.4	134.3	387.7	9.6	2.7
Pb-L9′	11						
Cont.-N11a″	14						
Cont.-N12	13 av.	18.5	16.4	160.3	524.7	11.5	3.2
Cont.-N13	10						
Cont.-S10	11	17.1	17.1	157.6	534.3	10.9	3.2

Pb—Lead-subjected embryos.
Cont.-N—normal controls.
Cont.-S—saline controls.
Cont.-W—window controls.

disturbance due to operation does slow down growth so that it takes a longer time for the embryo to reach the same somite number as the undisturbed control. But notwithstanding this delay, when the embryo does reach a given somite stage of development it is essentially the same size as its normal control of somewhat younger age. This is shown in the table. On the other hand, the leaded embryo is not only delayed in reaching normal somite count, but also when this is reached after the longer interval of incubation, it has failed to attain the dimensions normal for the somite stage of development. Hence it is obvious that a specific type of gross growth retardation is produced by lead.

SUMMARY AND CONCLUSIONS.

Our study of the effect of the lead ion on the development of the chick embryo has brought out the following facts:

1. Gross growth is retarded.
2. Somite growth is retarded to a degree greater than that exhibited by body length and width.
3. The head and optic anlagen are regions of particular sensitivity. Their differential development is markedly inhibited.

From the purely biological point of view these results are in line with the findings of Child (10) and his school as to the sensitivity of the head end of rapidly growing organisms to harmful influences, and with those of Stockard (11) as to the peculiar sensitivity of the optic anlagen.

It is almost too well known to need repetition that the head region and the somites of embryos are specific areas of intense growth by increase in cell number. Therefore, turning from the general to the particular, the differential retardation of these regions which is caused by lead, is evidence justifying the conclusion that it is areas of rapid growth by cell proliferation which are selectively inhibited by this metallic ion.

Credit for the drawings is gratefully given to Eleanor Paxson, artist to the Research Institute.

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STUDIES UPON THE EFFECT OF LIGHT ON BLOOD AND TISSUE CELLS.

II. The Action of Light on Erythrocytes in Vitro.

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(Received for publication, July 9, 1928.)

In the course of a series of studies on white blood cells exposed to the action of light, it was found that they underwent a rapid and extreme degeneration (Earle (4)). It has also been found that red blood cells, when exposed to light, undergo degenerative changes, the cell swelling, and finally undergoing hemolysis.

The fact that red blood cells of the beef may be hemolyzed through the action of light from the carbon arc, was shown by the work of Schmidt-Nielsen (10) in 1906. A similar hemolysis was also demonstrated in the same year by Busck (1), who used the blood of the rabbit and irradiated it with light from a carbon arc. Busck also demonstrated that the rays capable of causing this hemolysis were filtered out by glass, and therefore lay in the ultra-violet region of the spectrum. Busck considered that some of his experiments suggested that oxygen played a definite rôle in this hemolysis.

Dreyer and Hanssen (3) illuminated human erythrocytes by means of light from a Bangs lamp, having cooled silver electrodes, and found that, once hemolysis had begun, the number of cells hemolyzed each minute coincided with the number which would be hemolyzed if the reaction were a monomolecular one. In this work the red cells were suspended in saline, the cells being placed on a hemocytometer slide and covered with a quartz cover-slip, through which the light was directed.

Schmidt-Nielsen (11) irradiated erythrocytes of the beef by means of light from

* This work was begun with the assistance of a grant from The Henry Strong Denison Medical Foundation, and has been continued under a fellowship in medicine from the National Research Council. It is presented to the Graduate School Committee of Vanderbilt University in partial fulfillment of the requirement for a thesis for the degree of Doctor of Philosophy.

a carbon arc, and found that the light sensitized the cells to hypotonic solutions, instead of producing a true primary hemolytic action. He found that hemolysis of unirradiated cells did not occur until the salt content of the solution was reduced to about 0.6 per cent NaCl, whereas, after irradiation, continued hemolysis occurred when the salt concentration was lowered to 0.9 per cent NaCl. When the cells were placed in solutions of high salt content, however, this hemolysis did not occur.

Hasselbalch (5) had his attention called to the action of light on blood through the lessened oxygen capacity of blood which had been exposed to light. He showed that this diminution in oxygen capacity was the result of a transformation of hemoglobin into methemoglobin. Hasselbalch also found that while the light wave-lengths, most active in causing hemolysis, were those shorter than 310$\mu\mu$, there was some slight action by longer wave-lengths, transmitted by glass. He also found that this hemolysis, as a result of irradiation, occurred even in a high vacuum, and concluded that the presence of air was not essential for its occurrence.

Koeppe (6), using human blood, verified this action of the ultra-violet region of the spectrum in causing a transformation of hemoglobin to methemoglobin, and in causing an hemolysis of the red cells, but believed that this hemolytic action was limited to wave-lengths shorter than about 270$\mu\mu$. From his results he considered that it was the action of light on the shells of the red cells which caused their hemolysis. Koeppe also emphasized the observation that a relatively short irradiation of the red cells caused very slight immediate hemolysis, but that after the elapse of some time without further irradiation, this hemolysis was greatly increased. Koeppe considered that there was an oxidation of the lipoid material of the shell of the cell, during the actual process of irradiation, this oxidation occurring in the presence of oxygen, and resulting in the formation of acids which, in turn, caused continued hemolysis of the red cells after the irradiation had been discontinued. Koeppe was able to show the existence of some substance in the suspending fluid, in which red cells had been irradiated, which was able to hemolyze unirradiated red cells.

Koeppe (7) also demonstrated that samples of children's blood, drawn immediately after a 30 minute irradiation of the children with a quartz mercury vapor lamp, were markedly more susceptible to hemolysis by light than were samples drawn before the children had been irradiated.

Reed (9), irradiated the blood of the dog with the tungsten arc, the light reaching the blood by passing through the wall of a quartz tube inserted in the course of the carotid artery. He found that only minor changes occurred in the hemoglobin concentration of the blood, and in the erythrocyte count.

Clark (2) irradiated the ears of rabbits with light from the iron arc and also noted only minor changes in the hemoglobin and in the erythrocyte count.

Miles and Laurens (8) irradiated dogs 1 hour a day for 8 days, using the carbon arc as a light source, and found an initial increase in the red cells. This increase was later followed by a decrease.

Materials and Methods.

The observations were made on hanging drops of blood obtained from rabbits. A drop of blood was drawn by pricking an ear vein. A cover-slip was touched to this drop, removed, and given a slight jerk so that the blood adhering to the cover-slip was spread out into a streak. The cover-slip was then inverted over a hollow ground slide, and its edges sealed with "Salvoline." The culture was placed at once in a dark water-jacketed incubator kept at 39°. This entire procedure was carried out in dim light, and as none of the preparations was kept longer than about 3 hours, no special effort at asepsis was made. These preparations were made in groups of three or four, one preparation being irradiated and simultaneously observed, the other two or three in the set being kept in the dark as controls.

In the irradiation of these cultures, the same light source and optical system used in the work previously reported (reference 4; spectrum 4; Fig. 4) were employed. Irradiation was begun within 10 minutes after the preparation of the culture. The culture was kept under constant observation during the whole time of irradiation.

EXPERIMENTAL.

A representative culture showing the degeneration of the red cells under the action of light is presented below. Where reference to the degeneration of the leucocytes is made, discussion has been limited to the polymorphonuclear neutrophil, which is used as a typical example.

Experiment 1.

Culture 1572.—Irradiation was begun. The red cells were normal at this time: the leucocytes were moving around slowly. *30 minutes later:* The red cells were markedly swollen and appeared practically biplanar in profile. *40 minutes:* The red cells were even more swollen, the neutrophils were moving slowly and irregularly. *55 minutes:* Within the last 5 minutes a complete hemolysis of all red cells within the irradiated area had occurred. When examined in gross at this time the slide showed an area of complete hemolysis.

Culture 1573.—Control culture, kept in the dark. *60 minutes after inoculation:* The red cells were not swollen, nor were there any gross signs of hemolysis. The neutrophils were apparently quite normal and were moving around fairly rapidly.

The process of degeneration of the red cells, under the action of light, as observed in these cultures, took place as follows: In from 15 to 30 minutes after irradiation had begun, the red cells in the irradiated area of the culture showed marked swelling. At first they

looked merely a trifle thicker than normal, especially around their periphery. The swelling continued however, and the erythrocytes gradually lost their biconcave profile and became first almost biplanar in profile, then biconvex. In a short time they had become perfectly spherical, and also more turgid, as indicated by an increased resistance of the cells to deformation. However, in spite of the swelling and increased turgor, there was only a slight increase in the greatest diameter of the cells. This process of swelling required from 30–90 minutes.

During the early part of this process there was no appreciable liberation of hemoglobin. During the latter part the results were inconstant, but in the majority of instances there was a gradual liberation of hemoglobin from the cells. This appeared to take place from all of the cells simultaneously. Often however, there was no trace of such an hemolysis during the swelling process. In such instances, a short time after the cells had become spherical, there was a sudden liberation of large concentrations of hemoglobin from the cells into the surrounding solution. This liberation appeared to take place simultaneously from all of the cells, and it often took place so suddenly that while at the beginning of the liberation process, the cells were normal in color, 3 minutes later not a single pigmented red cell could be distinguished in the whole microscopic field. All had been reduced to achromatic shadowy shells, while the culture medium had a deep orange tinge from the liberated hemoglobin. This complete process of liberation required about 90–110 minutes.

In the cells of a few cultures another modification of the process of liberation of the hemoglobin was noted. In these, the release occurred very gradually and never went to completion, as the cells remained of a distinct red color. Upon continued irradiation, instead of further liberation of pigment, the red cells took on the appearance of having been coagulated. They became less translucent than the normal cells and their outlines became increasingly hazy.

Hanging drop preparations similar to those above described, were made in the course of some other experiments. In these, however, the blood was diluted with 5–10 volumes of unbuffered or buffered Locke's solution. Upon irradiation of these preparations a strikingly different picture of degeneration was noted. As before, the cells be-

came swollen, and finally spherical, but following this, instead of a partial hemolysis of the cells, accompanied by a coagulation, or a sudden, almost explosive hemolysis of all of the cells exposed to light, there occurred a complete hemolysis of the cells, one or two at a time. This process was continued until finally, in from 15 minutes to 4 hours after the initiation of hemolysis, every cell in the field appeared as only a colorless shadow or "ghost" cell. Typical protocols from this series may be seen in Experiment 3 (page 672).

In the actual process of liberation of the hemoglobin, in both the diluted and the concentrated blood, no change could be observed in the walls of the red cells, and there was certainly no sign of gross rupture. Apparently the liberation of pigment was due to some submicroscopic change, either in the cell contents, the cell wall, or both, this change being such as to allow the pigment to leak out through the cell wall.

The next point considered was whether the range of wave-lengths of the light rays causing this hemolysis coincided with the range causing the degeneration of the leucocytes, as described in the previous paper. To determine this, hanging drops of blood were employed, as in the preceding experiment, and the same light source was used as formerly. Wratten filters Nos. 29, 45 and 58, and a 5 per cent total transmission neutral filter were consecutively substituted for the Corning daylight glass in the optical system. Wave-lengths of light transmitted by each of the filters employed were very active in causing hemolysis of the red cells. Further, the rate of degeneration of the cultures when irradiated under any one of the three colored filters, was practically the same. Nor did the rate vary from the rate shown when the culture was irradiated without a filter or with a 5 per cent total transmission neutral filter.

In all of these preparations serum had been present in some quantity, and the question next came up as to whether its presence was a factor conditioning the appearance of this degeneration of the red cells under the action of light. In order to obtain data on this point, 1 cc. of blood was drawn from an unanesthetized rabbit, by cardiac puncture. This blood was immediately mixed with 15 cc. of unbuffered Locke solution and then centrifugated at low speed in order to separate the cells. In this way the red cells were washed eight

times. The cells were then suspended in 10 cc. of unbuffered Locke solution and hanging drop preparations were made as before. Upon irradiation of these hanging drops, complete hemolysis of the cells occurred as usual, except that it took place with greater rapidity than in the hanging drops of whole blood, and in a manner similar to that described for the irradiated cells of whole blood diluted with Locke solution. From this it would appear that serum is not essential for the degeneration of the red cells under the influence of light. The protocol of a representative culture of this series is presented below.

Experiment 3.

Culture 1625.—Washed red cells, suspended in Locke solution. Upon first examination the erythrocytes appeared normal. Irradiation was begun. *9 minutes later:* Every cell in the field had swollen until it was spherical. At this time two cells in the field had hemolyzed. *13 minutes:* About 70 per cent of the cells in the field had hemolyzed, the rest were swollen but showed no trace of hemolysis. *16 minutes:* There were only two unhemolyzed cells left in the field. *16½ minutes:* The last cell had hemolyzed. The control cultures kept in the dark showed no hemolysis.

In this laboratory, during the last 3 years, many thousands of supravital preparations of blood cells have been made and studied. Some of these have been exposed to light for a number of hours, but no striking or rapid degeneration of these smears, due to the action of light, has been noted. These supravital preparations were made by placing a drop of blood on a cover-slip and dropping the cover, blood surface down, onto a clean slide coated with neutral red or neutral red and Janus green, and sealing the culture with vaseline.

There being the possibility that a degeneration due to the action of light did occur in such a preparation, but that it had been overlooked, a group of such flat smear preparations were made, the blood being drawn from the ear vein of a rabbit. No dye was used on these slides. At the same time, a group of similar preparations were made, the only difference being that instead of inverting and sealing the cover-glasses on the usual plane slides, they were inverted over the wells of hollow ground slides, giving preparations such as those used in the last experiment. The edges of the cover-slips were sealed with Salvoline. Of these two sets of slides, half of each set was

irradiated with light from the source previously described, and the other half was kept in the dark incubator as a control. Data from representative cultures from these sets are given in Tables I and II.

TABLE I.

Time Required for the Degeneration of Leucocytes and Erythrocytes in Hanging Drop Preparations of Whole Blood.

Culture number	In light		In dark (control)	
	Time	Condition of cells	Time	Condition of cells
	min.		*min.*	
1051	180	r.b.c. swollen, hemolyzed; p. markedly degenerate		
1052			360	r.b.c. not hemolyzed; no data on p.
1053			360	r.b.c. not hemolyzed; no data on p.
1057	70	r.b.c. hemolyzed; p. rounding up, edematous		
1058			190	r.b.c. normal; p. normal
1058 A			190	r.b.c. normal; p. normal
1059	100	r.b.c. hemolyzed; p. rounded up, degenerate		
1060	50	r.b.c. swollen; p. rounded up		
1061			75	r.b.c. normal; p. normal
1062	65	r.b.c. swollen; p. rounded up		
1063			190	r.b.c. normal; p. normal
1317	120	r.b.c. swollen; p. rounded up		
1318			160	r.b.c. normal; p. moving very slowly
1319			160	r.b.c. normal; p. moving very slowly
Average...	100 min. or 1.7 hrs. r.b.c. swollen, hemolyzing p. swollen, hemolyzing		197 min. or 3.3 hrs. r.b.c. normal p. normal	

r.b.c...red blood cells.
p.....polymorphonuclear neutrophils.

Table I, Columns 2 and 3, shows that an average of 1.7 hours was required for the neutrophils present in the irradiated area of the hanging drop culture to round up. At this time, many of the cells showed a distinct hydropic degeneration. By this time also the red

TABLE II.

Time Required for the Degeneration of Leucocytes and Erythrocytes in Flat Smear Preparations of Whole Blood.

Culture number	In light		In dark (control)	
	Time	Condition of cells	Time	Condition of cells
	hrs.		*hrs.*	
1025	6.5	r.b.c. normal; p. moving slowly		
1026			6.5	r.b.c. normal; p. moving slowly
1063	7.0	r.b.c. practically normal; few rounded up; majority still motile		
1064			7.0	r.b.c. in slightly better condition than in No. 1063; p. as in No. 1063
1450	4.0	r.b.c. normal; many p. rounded up; some still motile		
1451			4.0	r.b.c. as in No. 1450; p. normal
1452			4.0	r.b.c. as in No. 1450; p. normal
1453	6.0	r.b.c. slightly shrunken; no gross hemolysis; p. slowly motile		
1454			6.0	r.b.c. normal; p. normal
1455			6.0	r.b.c. normal; p. moving slowly
1456			6.0	r.b.c. normal; p. moving slowly
1457	11.0	Some of r.b.c. hemolyzed, some swollen; hemolysis slight; p. rounded up		
1458			11.0	r.b.c. as in No. 1457; p. as in No. 1457
1459			11.0	r.b.c. as in No. 1457; p. as in No. 1457.
Average.....6.9		r.b.c. normal or slightly degenerated p. slowly motile; some rounded up	6.9	r.b.c. slightly less degenerated p. slightly less degenerated

r.b.c...red blood cells.

p.....polymorphonuclear neutrophils.

cells within the illuminated area were badly swollen and showed definite hemolysis, and in almost all cases free red cell pigment could be seen in solution in the culture medium.

Columns 4 and 5 of Table I show the results from similar cultures kept in the dark as controls. At the end of an even longer average time, 3.3 hours, there was no sign of a rounding up or of hydropic degeneration of the neutrophils of these cultures, nor was there any other sign of their degeneration. Further, even when the slides were examined after 4 hours, there was no sign of hemolysis of the red cells, except of those few cells injured in the preparation of the culture.

On the other hand, as shown in Columns 4 and 5 of Table II, at the end of 6.9 hours the neutrophils in the flat preparations kept in the dark, were moving around, though rather slowly, and the red cells showed only a very slight degeneration. The neutrophils in the flat preparations exposed to light were also still moving around, though very slowly, generally a little more slowly than in the control, while the red cells showed only slightly more degeneration than had been shown by those in the flat slide cultures held in the dark.

It seemed legitimate to conclude that this striking difference in the rates of degeneration of the blood cells on flat slides and in hanging drops, when irradiated, was due to the influence of some factor essential for the degeneration of both red and white blood cells when subjected to irradiation. In considering what this factor might be, there were three possibilities: (1) differences in evaporation from the two types of cultures, (2) differences in the thickness of the irradiated blood film, or (3) differences in the amount of air with which the culture was in contact.

An examination of the first of these, *i.e.* the influence of evaporation, showed that in the flat slide preparations the only marked evaporation which occurred was that which took place within the few seconds before the culture was sealed. On the hollow ground slide, however, in addition to this evaporation, there was also the evaporation from the hanging drop necessary to saturate the air in the hollow chamber of the slide. This chamber was found to have a volume of 0.25 cc., whereas the volume of the hanging drop of blood used, as measured from a volumetric pipette, was found to be about 0.005 cc. The saturation point of air for water vapor at 39° is about 0.00005 gm.

/cc., therefore 0.0000125 gm. of water vapor from the hanging drop would be required, to saturate the air in the well of the hollow slide. This represents an evaporation of about 0.25 per cent of the total volume of the drop. It is obvious that this additional evaporation is too small to constitute the factor responsible for the difference in the behavior of the two types of cultures under the action of light.

In considering the second possibility, that the thickness of the layer of red blood cells might constitute the essential difference in the two types of preparations, it was obvious that the hemolysis of the red cells and the degeneration of the white cells might be dependent on the greater absorption of light carried on by a relatively thick layer of cells, as in the hanging drop preparations. It might not occur at all, or to a very slight degree in films one cell thick, as in the flat slide preparations. This hypothesis was tested out by making a diluted suspension of red cells in unbuffered Locke solution, and irradiating hanging drops of these, as previously described. By this technique, films were obtained which were one cell thick, and which consequently had the same magnitude of light absorption as did the films on the flat slides. The concentration of serum in these cultures was lower than the concentration in the cultures of whole blood, but it has already been shown that the hemolysis of the red cells under the action of light, was not dependent on the presence or absence of such concentrations of serum (page 672). In these preparations, as in the hanging drops of undiluted blood, the hemolysis of the cells was striking, and showed conclusively that the essential difference between the behavior of the flat slide preparations and the hanging drop preparations of whole blood was not due to the thickness of the layer of blood cells.

On the other hand, while, owing to the marked reducing powers of the freshly drawn blood itself, the blood on the flat slide was under strongly anaerobic conditions within a very few minutes from the sealing of the culture, the hanging drop culture remained in contact with about 50 times its own volume of air. It seems logical to assume that this was the difference responsible for the divergent behavior of the two types of preparations when subjected to irradiation.

DISCUSSION.

The degeneration, or hemolysis, of the red cells under the action of light, as described in this article, accords with the results of previous workers, who have found that light does exert a marked hemolytic effect on red blood cells. In particular it confirms the work of Hasselbalch who found that this hemolytic action was caused by ultra-violet light, and also by light wave-lengths extending at least a short distance down into the visual spectrum. At the same time it extends Hasselbalch's work in showing that the rays active in causing this degeneration are distributed throughout the visual spectrum, lying in each of the three following zones: (*a*) 430$\mu\mu$–550$\mu\mu$; infra-red; (*b*) 475$\mu\mu$–630$\mu\mu$; 690$\mu\mu$– infra-red; (*c*) 600$\mu\mu$–infra-red.

In considering the relative activity of the blue, green and the red regions of the spectrum in causing this degeneration, it should be noted that the green filter transmitted about 23 per cent of the light reaching it, while the blue and red transmitted only about 5 per centand 6.6 per cent respectively. The time required to produce hemolysis by the action of light passing through any one of these three filters was, within the limits of error, the same. This fact appears to be open to two possible interpretations: (1) that through chance, the activity of the spectral zone transmitted by each of these filters, times its total transmission, is practically identical for the whole series of three filters, or (2) that within the range of light intensities used, different intensities produced little change in the period of time required to cause hemolysis of the red cells. That the second condition prevailed was shown by irradiation of the cultures through a neutral 5 per cent filter. With such a filter, the time required to produce hemolysis of the red cells was practically the same as when no filter, or any one of the three above described, was used.

If a comparison is made between the process of degeneration of the neutrophils under the action of light, as previously described (4), and the process of degeneration of the red cells, the similarity is evident. Both types of cells swelled to a marked degree. In the neutrophils this swelling often occurred to such a degree as to rupture the cells, and in the red cells it became so great as to result in an extrusion of cell contents (pigment), although this extrusion did not

occur through a gross rupture of the cell wall. It will be noted also, that the terminal stage of the degeneration in the neutrophils was often one of cell coagulation. As has been stated, in some instances the hemolysis of the red cells was incomplete, the cells assuming an opaque, coagulated appearance after having lost only a part of their pigment. It would seem that light had produced some degree of coagulation of the cells before all of the hemoglobin could escape, thus producing a condition in the red cells paralleling the terminal coagulation of the neutrophils.

One very striking point in regard to these experiments was the interrelation of the degeneration time of the red cells and of the neutrophils. During the whole process of the degeneration of the red cells, up to the time when the release of hemoglobin began, there was generally little or no change in the neutrophils. However, within a short time after hemoglobin began to appear in the surrounding medium, the neutrophils rapidly ceased moving and rounded up. Following this, the degeneration of the white cells was rapid, and often within 10 or 15 minutes the neutrophils were showing a marked hydropic degeneration.

Inasmuch as the red cells are heavily pigmented, whereas the white cells are practically achromatic, it appears natural that the former should, because of their greater light absorption, degenerate far sooner than the latter. That this is the case has been shown in these experiments, in which the red cells generally showed a marked hydropic degeneration before the white cells were visibly affected. At the same time it is possibly more than a coincidence that the degeneration of the white cells was often first noted immediately following the escape of hemoglobin from the red cells. The question is naturally raised as to what rôle, if any, the substances liberated from the red cells in such a degeneration may play in causing the degeneration of the white cells.

One rather striking point brought out by these experiments is the difference in the manner of hemolysis of the cells in whole blood and in blood diluted with unbuffered Locke solution. Upon irradiation of either, there was a gradual swelling of the cells. Following this swelling, in whole blood, the liberation of pigment often occurred with almost explosive rapidity, every cell in the field undergoing complete

hemolysis in just a minute or so. In cases where this rapid hemolysis was not found, there was apparently a gradual loss of hemoglobin from all of the cells simultaneously. This process sometimes required as long as several hours. In dilute cell suspensions however, after the cells had become spherical, the cells all hemolyzed one or two at a time, as described by Dreyer and Hanssen (3). While this difference was sharply defined, the data are at present too incomplete to allow any conclusion as to the factors responsible for it.

In considering the influence of serum on the hemolytic action of light on red blood cells, the hemolysis of the cells occurred either when serum was present only in traces, or in large amounts. While it has been impractical to study the action of light on cells freed from the last trace of serum, it would appear that the hemolysis is not dependent on the presence or absence of serum, although the serum may serve to retard the reaction.

In regard to the comparative behavior of the flat preparations and the hanging drop preparations of whole blood, when irradiated, the factors of evaporation, of the thickness of the blood film, and the concentration of the blood serum have been eliminated. The data indicate that the essential difference between the two types of preparations has to do with the amount of air with which the culture is in contact. In the hanging drop preparations the blood is in equilibrium with about 50 times its own volume of air, whereas, in the flat preparations, the blood is so sealed that it has no contact with the air.

While this conclusion supports the belief of Busck (1), that oxygen plays some rôle in the hemolysis of red cells under the action of light, it does not agree with the conclusions of Hasselbalch (5), who found that it occurred in the absence of air. Because of the many points of variation in the technique employed by Hasselbalch and that employed in this work, further data will be necessary before an accurate comparison of results can be made.

SUMMARY.

1. It was found that when hanging drops of whole blood, drawn from a rabbit, were subjected to irradiation from certain light sources, a striking degeneration of the white and of the red cells occurred. In this degeneration of the red cells there was (*a*) a preliminary period

of 15–30 minutes during which no effect was noted; (*b*) following this, there was a period during which the cells swelled and became first almost biplanar, then biconvex, and finally spherical. After a spherical shape had been assumed, the liberation of blood pigment from the cells began. This process progressed with varied rapidity, sometimes being very slow, and being incomplete; sometimes being of almost explosive rapidity. This liberation was not accomplished in either case through a gross rupture of the cells, but through the agency of some submicroscopic change produced either in the cell contents, the cell wall, or both. Following this liberation of pigment, in some cultures the red cells showed signs of coagulation, as did the white cells. In other cultures, however, the red cells were reduced to achromatic shadows.

2. When whole blood was diluted with unbuffered Locke solution and then irradiated in hanging drop preparations, the erythrocytes swelled, as in whole blood, but then, instead of an almost instantaneous hemolysis of every cell present, or a slow liberation of hemoglobin from each individual cell, all the red cells hemolyzed, one or two at a time, the hemolyzed cells being left as achromatic shadows.

3. This hemolysis of the red cells, as in the case of the degeneration of the white cells, occurred upon irradiation of the culture with white light, or with light lying in each of the three spectral zones of the visual spectrum, defined by Wratten filters Nos. 45, 58 and 29 respectively, as follows: (*a*) 430$\mu\mu$–550$\mu\mu$; infra-red; (*b*) 475$\mu\mu$–630$\mu\mu$; 690$\mu\mu$–infra-red; (*c*) 600$\mu\mu$–infra-red.

4. Within the range of intensities of light employed, there was little or no difference in the rate at which light of these three regions acted on the red cells. Further, this rate was the same as was the rate when the red cells were irradiated with no colored filter interposed in the optical path, or when a 5 per cent total transmission neutral filter was interposed.

5. It was shown that this degeneration of the red cells under the action of light was not dependent on the presence or absence of serum, with the possible exception of that trace which might have been adherent to the red cells even after repeated washing.

6. The white cells in the culture generally showed no changes until after traces of red cell pigment could be seen free in the surrounding medium.

7. On the other hand, no neutrophils were observed moving around for longer than a few minutes after the liberation of red cell pigment had occurred.

8. The liberation of substances from the red blood cells, as a result of irradiation of such preparations, may play some major rôle in the degeneration of irradiated white cells.

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STUDIES UPON THE EFFECT OF LIGHT ON BLOOD AND TISSUE CELLS.

III. The Action of Light on Fibroblasts in Vitro.

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Plate 17.

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INTRODUCTION.

In the course of a series of observations on the effect produced on leucocytes and erythrocytes by irradiation *in vitro* (1), it was found that these cells underwent an extreme and rapid degeneration when exposed to light of various regions of the visual spectrum. The data obtained were such as to suggest that substances liberated from the erythrocytes, during their degeneration under the influence of light, played some rôle in the degeneration of the leucocytes when exposed to light. For the further analysis of this problem it seemed essential to obtain the leucocytes free from erythrocytes. Inasmuch as attempts to do this were unsuccessful, it was decided to try the fibroblast, which cell was found to be fairly satisfactory. Further work, detailed in this article, has indicated that the presence of the red blood cells does play a major rôle in degeneration of the fibroblasts under the action of light of the visual spectrum.

Kiaer (3), in 1925, studied the action of light from a Kromayer quartz mercury vapor lamp on pure cultures of fibroblasts. He found that the light from this source exerted a definite impeding action on the growth of the fibroblasts in cul-

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ture, and that an exposure of from 30–60 minutes resulted in the death of the cells. Light from this source is very rich in ultra-violet wave-lengths and as these are known to produce striking biological effects, Kiaer's data cannot be considered as necessarily applying to the longer wave-lengths of light lying within the range of the visual spectrum, with which the previous articles of this series (1, 2) were concerned.

Material and Methods.

The fibroblasts used in this investigation were obtained from the hearts of embryo chicks of from 6–12 days incubation. In obtaining the tissue, the chick was removed from the egg and washed in Tyrode solution (pH 7.4). The heart was then removed and cut up in the same solution, using a pair of iridectomy scissors for this work. Fragments of the tissue were then transferred to cover-glasses by means of a platinum loop, and there covered with a drop of Tyrode solution (pH 7.4). The cover-glass was then inverted over the well of a hollow ground slide, and its edges sealed with Salvoline. This whole process was carried out aseptically in very dim light. The cultures were immediately set in a water-jacketed incubator in the dark and kept there at 38° until the fibroblasts had moved out on the cover-slip in a sheet of some size. This required from 10–60 hours. At the end of this time the cultures were divided into two groups; one group was irradiated, while the other group was kept in the dark incubator as a control.

The cultures which were to be irradiated were all given a careful examination at the beginning of irradiation and were discarded at once if any trace of cellular degeneration was present. Inasmuch as the least examination of the controls would have vitiated the experiment, these could not be given a preliminary examination.

The light source and optical system used in this work were the same as those used in the previous work (1). Spectrograms of this light source, and of this light source with the special filters described later, have already been shown (Earle (1), spectra 4, 5, 6 and 7, Fig. 4). In all of these experiments, irradiation and microscopic examination of the cultures were carried out simultaneously.

EXPERIMENTAL.

In the preliminary work on this problem, an attempt was made to irradiate the fibroblasts under conditions as similar as possible to those under which the white cells degenerated. The factors which had to be taken into consideration were, (1) that the white cells themselves were present (2) that red cells were present in varying numbers, (3) that even in cultures of cells in saline, at least traces of serum were present and (4) that air was present in large volumes compared with the actual volume of the explant.

In order to secure cultures of fibroblasts in which these conditions prevailed, red blood cells were obtained by puncture, with a capillary pipette, of one of the extra embryonic blood vessels of an embryo chick. These red cells were washed with three changes of Tyrode solution having a pH of 7.4. Cultures were then made using tissue from the heart of the same chick. These cultures were inoculated, as described above, except that a few of the washed red blood cells were also added. Some of these cultures were irradiated while the others were kept in the dark as controls.

An examination of these irradiated cultures showed conclusively that under these conditions the light did cause an extreme degeneration of the fibroblasts. Beginning at about 15–30 minutes after the irradiation was started, the refractive index of the fibroblasts showed a gradual but distinct increase. Normally the fibroblasts in culture have such a low refractive index as to be seen only with difficulty, while the nuclei are exceedingly difficult and often impossible to discern. In these irradiated cultures, however, the refractive index of the fibroblasts increased so greatly that the cells and all of their processes could be outlined with ease. At the same time, the nuclei also became sharply defined.

After the lapse of from 30–140 minutes, vacuoles of low refractive index began to appear throughout the cytoplasm of the cells. These vacuoles did not appear to originate in any single region of the cells, nor did they generally seem to begin as minute sharply defined droplets, increasing in size. Rather, in some region where the cytoplasm had appeared homogeneous, there appeared a small area with a very slightly lower refractive index than the surrounding region, but at first almost indistinguishable from it. The optical distinction between this area and the surrounding region increased rapidly, however, until the droplet appeared as a clear area of low refractive index, a "punched out" looking area in the cell, surrounded on all sides by the more highly refractive cytoplasm. These vacuoles were of all sizes and occurred in different numbers in different cells. When irradiation was continued for 3 or 4 hours, practically every cell in the culture was densely crowded with them. Upon staining the cultures supravitally with neutral red, they took up some of the dye, and were colored orange-red by it. They showed no reaction with Sudan III,

or with osmic acid. From these staining reactions and from their low refractive index, it would seem that their chief content was probably water. About the time the vacuoles began to appear, the cells often showed some tendency to round up. If irradiation was continued until the cells were crowded with vacuoles, this rounding up was almost constant in its appearance, and often occurred to a very marked degree (Fig. 1).

A modified form of this degeneration was frequently seen in these cultures. Instead of the formation of discrete vacuoles in the cytoplasm of the cells, the viscosity of the cytoplasm showed a great decrease, as indicated by the marked increase in the amplitude of brownian movement of intracellular particles. The cells began swelling very rapidly, and, as a result, became almost spherical. None of these cells was ever seen to burst as a result of this swelling.

In the unirradiated fibroblasts in tissue culture, there were generally a few highly refractive droplets staining with Sudan III or with osmic acid, and obviously of a lipoid nature. At the end of several hours of irradiation, these droplets were often perceptibly increased, both in size and in number. The dominant features in this degeneration, however, were the change in the refractive index of the cell and the formation of vacuoles throughout its cytoplasm.

The whole process of degeneration as above described, often took place within 1 hour, and never required longer than 3. This degeneration was remarkably constant in its appearance, although in some cultures it appeared in the modified form previously described. In this modified form the changes seen appeared to be identical with those seen in the polymorphonuclear neutrophils under the action of light.

The protocols from a representative culture of this series, and its controls are here shown as Experiment 1.

Experiment 1.

Culture 801.—This culture was planted from the heart of an 11 day embryo chick. *16 hours after inoculation:* Irradiation was begun. At this time the fibroblasts had grown out in a sheet. The cells appeared normal; their refractive index was low, and a few fine highly refractive droplets were scattered through them. There was no sign of vacuoles in any of the cells. *40 minutes later:* A marked in-

crease in the refractive index of the cells had occurred; details of cell boundaries and nuclei could be made out. At this time some of the cells showed a single vacuole from one to four times as large as the nucleus. *100 minutes:* Many cells showed definite vacuoles, and quite a few were filled with them. In most of the cells the vacuoles were unmistakably of cytoplasmic origin, some of them originating far out in the pseudopodia. *300 minutes:* Practically all of the cells were filled with vacuoles. In this respect there had been little change in the last 150

TABLE I.

Experiment 2.

Time Required for the Degeneration of Fibroblasts in Vitro When Irradiated by Light Transmitted by Various Filters.

Culture number	Age of chick	Organ from which culture was taken	Age of culture	Time required for degeneration			
				No filter Transmission 100 per cent	Filter No. 45 (blue)	Filter No. 58 (green)	Filter No. 29 (red)
	days		*hrs.*	*min.*	*min.*	*min.*	*min.*
829	8	Heart	18			120	
831	8	Heart	27		630		
832	8	Heart	39				300
853	12	Heart	18	195			
854	12	Heart	About 26	160			
858	12	Heart	39				780
859	12	Heart	51				570
860	12	Heart	60	165			
897	9	Heart	35			130	
921	8	Intestine	14	145			
922	8	Intestine	About 16			90	
931	8	Intestine	About 21	160			
936	8	Intestine	18	100			
937	8	Intestine	20			70	
1579	8	Heart	About 16		600		
Average.....				158	615	102	550

minutes. The cells showed distinct signs of rounding up. The preparation was photographed at this time (Fig. 1).

Cultures 805 and 807.—Control cultures. Kept in the dark incubator. *21 hours after inoculation:* The fibroblasts appeared normal. They were of low refractive index, and were very difficult to see. There were only a few fine droplets in the cells, and no vacuoles were present.

The attempt was next made to see if, as in the case of the leucocytes, the degeneration of the fibroblasts was caused by wave-lengths of light

lying throughout the range of the visible spectrum. Accordingly, cultures were inoculated and, as in the case of the experiments on the white blood cells (1), were irradiated through Wratten filters Nos. 45, 58 and 29. The time required for the fibroblasts in each culture to undergo degeneration when that culture was irradiated by light transmitted by any one of these three filters is shown in Table I. This table also shows the time required for the degeneration of control cultures irradiated with the same light source but with no Wratten filter inserted in the optical path. Although not presented in this table, other control cultures, inoculated as above but kept in the dark, remained normal.

In preparing this table, the degeneration time for any culture was arbitrarily defined as the time required for the appearance of a marked increase in the refractive index of a majority of the fibroblasts in the microscopic field, accompanied either by the appearance of many vacuoles, in the cytoplasm of the cells, or by a marked tendency for the cells to round up and to show a greatly increased amplitude of brownian movement of their intracellular particles. It may be seen from this table that wave-lengths of light transmitted by any one of the three filters used caused the typical degeneration of the fibroblasts.

The next question considered was the possible mechanism of this action of light on the fibroblasts. In this consideration, three possibilities arose. The degeneration of the fibroblasts under irradiation might have been due to, (1) an intrinsic action of light on these cells, or (2) an intrinsic action of light upon the red cells with the formation of toxic substances, which in turn produced a degeneration of the fibroblasts, or (3) to a sensitization of the fibroblasts by some substance liberated from the red cells, with a subsequent action of light on the sensitized fibroblasts.

In order to determine whether the red blood cells did play a rôle in the degeneration of fibroblasts under the influence of light, cultures were prepared as before, except that this time no red cells were added to the culture. These cultures were then placed in two groups, one group being kept in the dark as a control, while the other was irradiated. A further control was kept on these cultures by irradiating similar cultures containing fibroblasts and red cells. All the cultures containing red cells and exposed to the action of light showed a typical

degeneration of the fibroblasts. In one group of eight cultures irradiated, every culture showed a typical extreme degeneration in less than 3 hours. On the other hand, of five cultures, supposedly containing no red blood cells, one showed the typical degeneration only after irradiation for 13 hours. The other four showed only minor degenerative changes at the end of from 13–24½ hours of irradiation. These minor changes consisted of an increase in the refractive index of the cells and the rounding of some of the sharper protoplasmic processes. In these cultures there was no indication whatsoever of any swelling of the cells, of any increase in the brownian movement of intracellular particles, nor of any formation of vacuoles within the cytoplasm. This contrast, between the behavior of the cultures containing red cells and that of the cultures containing no red cells, was augmented by the fact that at the close of the irradiation of the cultures containing no red cells, even some of the controls which had been kept in the dark, were showing traces of degeneration. In every instance, however, this degeneration of the controls was markedly less than that in the cultures kept in the light.

A protocol of a representative culture of this series is presented below as Experiment 3.

Experiment 3.

Culture 823.—Inoculated from the heart of an 11 day embryo chick. *24 hours after inoculation:* Irradiation was begun; the cells appeared normal at the time although they contained a good many fine droplets of fatty material. *100 minutes later:* There had been no change except a very slight increase in the refractive index of the cells. *270 minutes:* There had been no change and the cells appeared normal. *15 hours:* The cells showed a somewhat higher refractive index than was normal, and their nuclei were sharply defined. They were not rounding up and showed no vacuoles or swelling. *17 hours:* There had been little change. A representative cell was photographed at this time (Fig. 3).

DISCUSSION.

There was a definite similarity between the type of degeneration seen in the fibroblasts and that observed in the erythrocytes and in the leucocytes. The similarity between that shown by the fibroblasts and by the neutrophils was particularly striking. No formation of discrete vacuoles of large size has been observed in the neutrophils,

probably owing to their more fluid cytoplasm and its consequent more general liquefaction. It should be noted, however, that sometimes vacuole formation in the fibroblasts was so slight that no vacuoles could be made out in the unstained cells, the cytoplasm undergoing just such a liquefaction as occurred in the neutrophils. This liquefaction was indicated by a tremendously increased amplitude of brownian movement of particles within the cells. When this occurred the cells rounded up, became almost spherical, and swelled rapidly. Time and again, fibroblasts were seen in this state, and if the complete process of degeneration of the individual cells had not been followed, they would have been indistinguishable from the swollen polymorphonuclear neutrophils seen in the cultures of blood which had degenerated under the influence of light (Earle (1), Fig. 1).

It was found, as in the case of the leucocytes and the erythrocytes, that light of wave-lengths lying within the three zones (*a*) 430$\mu\mu$–550$\mu\mu$; infra-red; (*b*) 475$\mu\mu$–630$\mu\mu$; 690$\mu\mu$–infra-red; (*c*) 600$\mu\mu$–infra-red, was active in causing this degeneration of the fibroblasts. The relative activity of these three zones for the erythrocytes is apparently not the same as for the fibroblasts, for the intensities of light employed. The light transmitted by each of these three filters caused an hemolysis of the red cells within practically the same time, and within the same time that was required by white light of even greater intensity. It was found, however, that when either the blue filter No. 45 (less than 5 per cent total transmission) or the red filter No. 29 (total transmission about 6.6 per cent) was placed in the beam of light irradiating the fibroblasts, the time required for the degeneration of the cells was much greater. A similar, though a less striking phenomenon was also noted in the cultures of leucocytes previously irradiated (1). This difference between the leucocytes and the fibroblasts on the one hand, and the erythrocytes on the other, is most interesting, although at present no explanation for it is offered.

Another interesting point brought out by these data is that when irradiated through the green filter, the fibroblasts consistently seemed to show a more rapid degeneration than when irradiated with white light (100 per cent transmission). This was also found true for the cultures of irradiated leucocytes (1). Before any definite conclusions are drawn on this point, however, these data should be verified and extended by means of a more accurate quantitative technique.

It was found that the fibroblasts, in cultures to which no red cells had been added, had degenerated much less at the close of from 13–24 hours of irradiation than they had at the end of from 2–3 hours irradiation when red cells were present. This observation indicates that the red cells played some major rôle in the degeneration of the fibroblasts. In these cultures, supposedly containing no red cells, the tissue was carefully washed, and red cells were certainly very few in number. However, a very few red cells were seen in the culture which showed the typical degeneration at the end of 12 hours. In the other cultures it is quite possible that either isolated red blood cells or traces of the substances liberated from disintegrated red cells were present. For this reason, it is impossible to say that even the traces of degeneration seen in these cultures were due to an action of light directly on the fibroblasts alone. Certainly any action of light of the visual spectrum on the fibroblasts alone was exceedingly slight, its major influence being conditioned by the presence or absence of red blood cells.

Whether the presence of the red cells is also essential to the degeneration of the leucocytes under the action of light has not as yet been determined. There are, however, several facts which indicate that it plays some rôle. In tissue cultures of leucocytes it was noted that, for any one light source, there was a marked variation in the time required for the degeneration in different cultures of any one series. In these cultures, there was also a marked variation in the number of red blood cells present. Furthermore, in hanging drops of whole blood, the degeneration of the leucocytes rarely occurred before there were marked signs of degeneration of the erythrocytes, and it was never delayed more than a few minutes after the hemolysis of the erythrocytes had begun.

SUMMARY.

1. In the presence of autogenous red blood cells, fibroblasts, grown *in vitro* from the heart and intestine of embryo chicks of from 6–12 days incubation, underwent a rapid degeneration when exposed to light of the visual spectrum.

2. In this degeneration, the cells showed a marked increase in refractive index, and a massive formation of colorless vacuoles of low refractive index. These vacuoles gave no reaction with osmic acid

or Sudan III, but took up neutral red. Later in the degeneration the cells showed marked signs of rounding up and of coagulation.

In some cases this degeneration process was modified in such a manner that, instead of the formation of vacuoles, the whole cytoplasm became much less viscid and the cells swelled greatly and became spherical.

3. The similarity of this degeneration to that shown by the erythrocytes, and more particularly, to that shown by the polymorphonuclear neutrophils, under the action of light, is pointed out.

4. Degeneration of the fibroblasts occurred when the cells were irradiated by light of any one of the following wave-length zones: (*a*) 430$\mu\mu$–550$\mu\mu$; infra-red; (*b*) 475$\mu\mu$–630$\mu\mu$; 690$\mu\mu$–infra-red; (*c*) 600$\mu\mu$–infra-red.

5. This degeneration was much slower for the cells irradiated through the blue or red filters of less than 5 per cent, and less than 6.6 per cent, respective total transmissions. When irradiated through the green filter of 23 per cent total transmission it was much more rapid, and even more rapid than for cultures irradiated with white light of 100 per cent total transmission. These data were obtained on a short series of cultures and must be considered only as suggestive.

6. In the apparent absence of red cells, the fibroblasts underwent only a slight degree of degeneration after irradiation lasting from 13–24½ hours. It is possible that even this minor degeneration may depend upon the presence of traces of disintegration products of red cells in the culture. Much more advanced degenerative processes were obtained, when many red cells were present, after only 3 hours irradiation.

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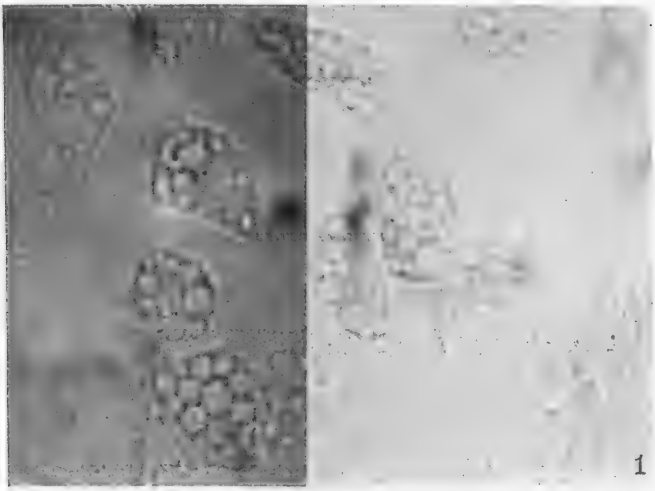
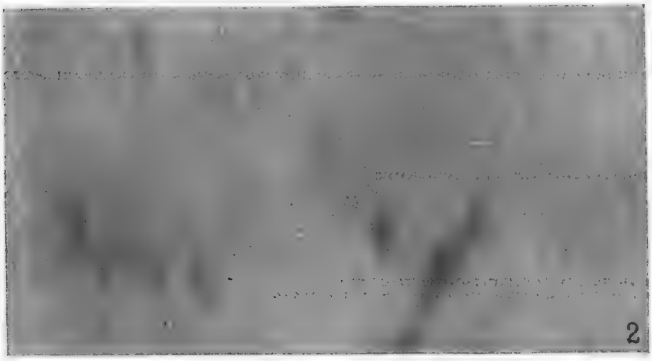
EXPLANATION OF PLATE 17.

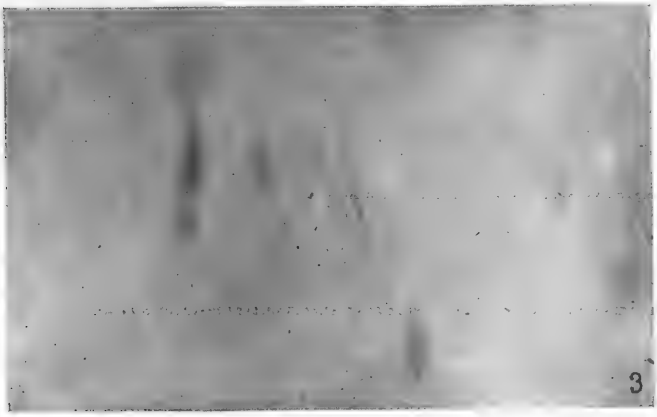
FIG. 1. Culture 801, × 1000 (see text, page 687). Fibroblasts taken from the heart of an 11 day chick embryo, and inoculated with erythrocytes present in the culture. Irradiated for 5 hours. Photographed so that half of the photographic plate was given a longer exposure than the other half. The fibroblasts show a striking increase of refractive index. There was a marked rounding up of the cells and the cytoplasm was filled with vacuoles.

FIG. 2. Culture 820, × 1000. Fibroblasts taken from the heart of an 11 day chick embryo, and inoculated in culture, no erythrocytes being present. Photographed 52 hours after inoculation of the culture and after 13 hours of irradiation. Note that while the refractive index of the cells is somewhat greater than normal, and that their cytoplasm is somewhat more granular than normal, there is no sign of rounding up of the cells, nor are there any vacuoles visible in the cytoplasm.

FIG. 3. Culture 823, × 1000. Fibroblasts taken from the heart of an 11 day chick embryo, and inoculated in culture, no erythrocytes being present. Photographed 40 hours after inoculation and after 17 hours of irradiation. Note that there is some tendency for the cell to round up; its nucleus is clearly outlined, and the refractive index of the cell is slightly increased. There is far less degeneration than was shown in Culture 801, Fig. 1, however.







(Earle: Effect of light on blood and tissue cells. III.)

UNIV. OF

EXPERIMENTAL PNEUMONIA IN GUINEA PIGS.

I. The Effect of Certain Toxic Autolysates of Pneumococci.

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Plates 18 and 19.

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Much work has already been carried out on the experimental production of pneumococcus pneumonia in animals. Important publications on the subject are those of Wadsworth (1) on the production of pneumonia in rabbits; of Lamar and Meltzer (2) and Meltzer and Wollstein (3) in dogs; of Cecil and Blake (4) in monkeys; and of Stillman and Stillman and Branch in mice (5–7).

It is logical to suppose that in order to produce pneumococcus pneumonia this organism must be made to grow in the lung. It has, however, been the experience of all workers that, in the case of the smaller laboratory animals—rabbits, guinea pigs, or mice—pneumococci will not infect the lung tissue save in exceptional instances, no matter how they are injected. Thus Wadsworth found that rabbits injected intratracheally with virulent or non-virulent pneumococci either failed to become infected or died with pneumococcus septicemia, but without pneumonic lesions. Stillman had the same experience in his attempt to produce pneumonia in rabbits by the inhalation method. In the case of mice also Stillman found that virulent pneumococci, though inhaled into the lower respiratory tract, disappeared within a few hours without calling forth a pneumonic reaction. Even in alcohol-intoxicated mice in which the pneumococci persisted in the lung for a longer period than in unintoxicated mice and in which fatal septicemia often followed, localization with production of pneumonia was rarely observed.

Wadsworth's negative results in attempting to produce pneumonia

in normal rabbits led him to repeat the experiments in systemically or locally predisposed rabbits. In this he was unsuccessful as was Stillman in similar experiments in mice. However, by provoking a partial immunity in rabbits, Wadsworth did succeed in regularly causing pneumonic lesions and recently Stillman has also succeeded in producing lobar pneumonia in partially immunized alcoholized mice exposed to an atmosphere of virulent pneumococci.

The obvious conclusion that emerges from this work on the production of pneumococcus pneumonia in the smaller laboratory animals is that under ordinary conditions, the pneumococci will not grow in the lungs of these animals and without such growth pneumonia is not produced. If this be true, one may imagine that in pneumococcus pneumonia in animals, and in man, there is a hypothetical substance or substances, perhaps produced in the growth or autolysis of the pneumococci, which by its action upon the lung tissues or fluids enables the pneumococcus to establish itself and to multiply, and in so doing to cause pneumonia.

In a previous communication (8) it was shown that Berkefeld filtrates of certain anaerobically produced autolysates of Pneumococcus I or Pneumococcus II cause necrosis when injected intracutaneously into guinea pigs. In the course of this work it was discovered that these autolysates when injected intratracheally were highly toxic, and that when introduced in this manner together with living pneumococci, the growth of the organisms in the lung became established. From these observations it seemed possible that such toxic filtrates might contain the hypothetical poisonous substances referred to above, and this report gives the experiments which were conducted in the study of this problem.

The experiments here reported refer (1) to the effect of toxic autolysate alone upon the lungs of guinea pigs, (2) to the effect of diluted toxin added to living pneumococci, in establishing the growth of the organism in the lungs. In this second group of experiments, controls were given of equivalent doses of (*a*) toxic autolysate alone, and (*b*) living pneumococci without autolysate.

We will discuss the biological and immunological properties of the lung-toxic substance or substances and compare these properties with those of the necrotizing poison in the autolysates in a later publication.

EXPERIMENTAL.

Intratracheal Injection of Toxic Autolysates Alone.

Preparation of Toxic Autolysates.—The method used in preparing the toxic autolysates was the same as that previously described (8).

Virulent Pneumococcus Type I or Type II was grown on double strength veal infusion broth containing 4 per cent Witte peptone. After 18 to 24 hours growth, and before any appreciable autolysis had taken place, the broth cultures were chilled and then centrifuged at high speed. After centrifuging the tubes were chilled, the supernatant fluid pipetted off carefully, and the pneumococci taken up in a quantity of freshly boiled and chilled broth equal to that of the combined sediment and supernatant fluid remaining. For the pneumococcus sediment from 100 cc. of broth culture, there should be in all approximately 1.5 cc. of fluid. The pH of the pneumococcus suspensions was brought to 7 or 7.2 and cultures made to be sure that only pneumococci were present in the preparation. Sometimes 0.25 per cent phenol was added to the preparations. The suspensions were distributed into narrow test-tubes, which were then chilled for at least 30 minutes. After this, any bubbles present on the surface of the suspensions were gotten rid of with a hot platinum loop and heavy vaseline seals added to all of the tubes. The tubes were left at room temperature in the dark at 22–24°C. for 2 to 5 days and then placed in the ice box. Before filtering, the autolysates were centrifuged, iced, the seals opened, and the clear supernatant fluid passed through a well iced Berkefeld filter. This filtrate contains the poison which is highly toxic when injected intratracheally. The filtrate was kept in the ice box under vaseline seal until used. It was necessary to keep the preparations chilled when they were exposed to the air, otherwise they became oxidized and the toxicity disappeared.

(*A*) *Results of Intratracheal Inoculation of Undiluted Toxic Autolysates Alone.*

It was found that strong necrotizing filtrates invariably produced marked symptoms followed by death when injected intratracheally in 0.2 cc. amounts in guinea pigs of from 180 to 300 gm. weight. The toxicity for the skin and for the lungs appeared to run parallel; that is, a filtrate which produced marked necrosis when injected intracutaneously was also extremely toxic when injected intratracheally into guinea pigs, while a filtrate of weak necrotizing activity caused only slight symptoms when injected intratracheally and the guinea pigs usually survived. Although there were individual differences in the reaction of guinea pigs even of the same weight after the injection of

a strong toxin, 0.2 cc. of the undiluted poison usually killed them in less than 3 hours, and, with three exceptions, in less than 24 hours. Guinea pigs almost invariably survived the intratracheal injection of strong toxin diluted 1 to 2 or 1 to 4 with broth.

1. Guinea Pigs Dying within 1 to 2 Hours after Injection.

Symptoms and Pathology—Within 5 minutes of the intratracheal injection of the toxic autolysates the guinea pigs showed marked dyspnea which continued until death. In the pigs which died in less than 6 hours, tonic and clonic muscular contractions and a bloody serous nasal discharge were noted before death. At autopsy, the lungs of the guinea pigs which died in less than 6 hours were greatly distended, very heavy, and mottled deep pink throughout. The cut section of these lungs centrally was extremely wet, deep red, and apparently airless.

Histological Findings.—Eight guinea pigs dying within 1 to 2 hours after injection were studied. All of these showed severe and definite pulmonary lesions: intense congestion, profuse alveolar hemorrhages, edema, stasis of leucocytes in the alveolar capillaries, and the presence of sparse numbers of emigrated leucocytes in the alveoli. The peribronchial tissues also were edematous, and loosely infiltrated with wandering cells. Fibrin was not conspicuous.

2. Guinea Pigs Surviving for 18 to 72 Hours after Injection.

In this group, $\frac{2}{3}$ to $\frac{3}{4}$ of both lungs was found at autopsy distended, heavy, deep red, and consolidated. There was a varying amount of normal tissue at the margins of some of the lobes. The cut surface of the red portions appeared completely solid. This group included animals injected with weak autolysates or with strong autolysates diluted 1 to 2 or 1 to 4 with broth and killed with ether at varying intervals after the injection. At autopsy, the appearance of the lung was similar to that described above save that the consolidated areas were concentrated in the hilum regions. It should be emphasized that the color of the pneumonic areas in all the animals injected with toxic autolysate alone—and over 100 have been examined—always was deep red and never even tinged with gray.

Histological Findings.—Fourteen animals of this group were studied. All showed patchy but quite extensive areas of pneumonic consolidation. The alveolar exudate in the earlier stages consisted of well preserved polymorphonuclear leucocytes, numerous red blood cells, and occasional large mononuclears. Fibrin was not abundant. There was swelling of the alveolar cells, necrosis in some areas of the alveolar wall with occasional formation of hyaline membranes. Edema of the peribronchial tissues and alveoli was less marked than in animals dying in the first few hours and massive hemorrhages were not present.

In animals killed after the 2nd day there were still pronounced pneumonic lesions, the ductuli alveolares were dilated and inflamed, and small pneumonic patches were scattered through the tissues; edema was still quite marked.

On the 3rd day the lesions showed evidence of regression, leucocytes were less numerous and undergoing fragmentation and autolysis. There was swelling and active proliferation (mitoses) of alveolar epithelium and capillary endothelium. In one animal the peribronchial lymphoid tissue appeared hyperplastic.

Results from Intratracheal Injections of the Following Preparations in 0.2 Cc. Doses.

B. Equal parts of pneumococcus cultures and toxic autolysates (undiluted or diluted 1 to 2).

C. Equal parts of pneumococcus cultures and broth.

D. Equal parts of toxic autolysates undiluted or diluted 1 to 2.

In every experiment in which pneumococcus cultures and toxic autolysates were injected together, controls of the same quantity of the pneumococcus culture alone (C) and the same quantity of the toxin preparation alone (D) were always injected separately into other guinea pigs.

Pneumococcus Strains Used.—The strains of Types I and II of the pneumococcus used in this work were the same as those used previously. Both strains when injected intraperitoneally were very virulent for rabbits, mice, or guinea pigs.

In these experiments we used only those filtrates which would kill guinea pigs within 24 hours, in undiluted doses of 0.2 cc. when injected intratracheally.

Technical Procedure.—18 to 24 hour broth cultures of Pneumococcus I and Pneumococcus II were used in this work. When there was profuse growth, the culture was diluted with broth to obtain the slight clouding desired. The pneumococcus culture was kept in ice water throughout the experiment.

Immediately before inoculation the tube containing the toxic filtrate was chilled, the vaseline seal opened, and the filtrate pipetted into a narrow chilled tube. This tube was kept in ice water throughout the experiment. Just before the injection, 0.2 cc. of the pneumococcus culture and 0.2 cc. of the toxin were pipetted into another iced tube, mixed, and taken up into an iced syringe; 0.2 cc. of this mixture was then immediately injected intratracheally into a guinea pig of 180 to 300 gm. A fresh mixture was always prepared before each injection, and the same precautions as to chilling, etc., were carried out with both control preparations of pneumococcus cultures and broth (C) and of toxic autolysate and broth (D).

(*B*) *Results From the Intratracheal Injection of Pneumococcus Culture and Toxic Autolysates.*

Forty-five guinea pigs were injected with mixtures of toxin and pneumococcus cultures. With one exception all died with pneumonic lesions and positive lung cultures. These forty-five guinea pigs may be conveniently grouped as follows:

1. 8 guinea pigs (18 per cent) which died in 6 hours or less.
2. 35 guinea pigs (77 per cent) which died in from 18 to 72 hours.
3. 2 guinea pigs (5 per cent) which survived longer than 3 days.

In this series are included a certain number of animals which were injected intraperitoneally 18 to 24 hours previous to the intratracheal injection of pneumococcus culture and autolysate with normal serum, either rabbit or horse, or with certain heterologous antipneumococcus horse sera. It will be shown in a later report that none of these sera had an appreciable effect upon the subsequent intoxication and infection caused by the intratracheal injections of mixtures of toxic autolysates and pneumococcus cultures.

1. Guinea Pigs Dying within 6 Hours after the Intratracheal Injection of Pneumococcus Culture and Toxic Autolysate.

These guinea pigs showed the same symptoms as those which had been injected with 0.2 cc. of a strong toxin alone. They were markedly dyspneic directly after the injection and continued in this condition

until death. At autopsy, the appearance of the lungs of these animals was also similar to that found in the guinea pigs which had been inoculated with toxin alone. Cultures from the lungs always gave a growth of the pneumococcus, and positive cultures were obtained from the heart blood in five of eight pigs. We believe that these animals were exceptionally susceptible to the toxin and probably would have died had 0.2 cc. of toxin diluted 1 to 2 been inoculated without pneumococcus culture.

Histological Findings.—Five animals (8-68, 7-59, 10-13, 43-74, and 7-07) of this group were studied histologically. In the two which died after $1\frac{1}{4}$ and $1\frac{1}{2}$ hours respectively, there had already developed loose areas of consolidation about the large bronchi in the hilus region. In these pneumonic patches the alveoli contained an exudate with fairly abundant polymorphonuclears; red corpuscles were very numerous; there was a slight amount of delicate fibrin. About the larger vessels and bronchi the tissue was intensely edematous and infiltrated with wandering cells, including many polymorphonuclears. The lymphatic vessels were distended. In the unconsolidated portions of the lung the congestion was intense; there were hemorrhages and moderate alveolar edema. Pneumococci were not found at this stage.

The remaining three animals of this group, dying 2, 3, and $5\frac{1}{2}$ hours after injection, showed virtually the same lesions, save that there was extension of the pneumonic areas, and an increased amount of fibrin. At this time, however, pneumococci were already present in considerable numbers, and in one, which died after 2 hours, an amazing multiplication of the bacteria had occurred.

2. Guinea Pigs Dying in from 18 to 72 Hours after the Intratracheal Injection of Pneumococcus Culture and Toxic Autolysates.

These animals did not appear sick or appeared only slightly sick after the injection. On the following day, however, they were all extremely ill, suffering from severe dyspnea which became progressively worse until death. At autopsy, from $\frac{1}{3}$ to $\frac{2}{3}$ of the total volume of both lungs was found enlarged and consolidated. The lower lobes were most frequently affected but consolidation was often present also in the middle and upper lobes. In all but one case both lungs were involved; in this instance, however, the entire right lung was

consolidated, and the left normal throughout. The pneumonic areas were deep red, yellowish gray, or greenish gray, the gray portions adjoining the hilum and the red portions surrounding them. The normal lung tissue was always at the margins of the lobes. The cut surface of the gray areas was yellowish gray and granular. In several of the guinea pigs which died in approximately 2 days, there was a cellulitis of the chest or abdominal walls. Cultures of the heart and lungs of this group always showed profuse growth of pneumococci.

Histological Findings.—Thirty-one guinea pigs of this group were studied. Although there were slight individual variations as regards the extent of the lesions, the pathological changes were on the whole surprisingly uniform. In almost all instances the greater part of both lungs was in a state of pneumonic consolidation. As was evident from the gross appearance, whenever a portion of the lobe remained unconsolidated, it was that at the margins, or distal from the hilic region.

In the earlier stages, the picture was in all essentials identical with that in the corresponding stages of lobar pneumonia in man. The alveoli were filled with an exudate composed predominantly of polymorphonuclear leucocytes; large mononuclears were not abundant, and when present could not readily be distinguished with the methods used from the exfoliated epithelium. The alveolar cells still attached to the septal wall were greatly swollen, and their cytoplasm vacuolated. Fibrin was irregularly present, but on the whole less abundantly and conspicuously than in human pneumonia. The capillaries in the earlier stages were engorged with well preserved erythrocytes. With the progress of the lesion, and the passage of the red blood cells into the alveoli, they frequently appeared collapsed and empty. Their basement membrane often seemed swollen and thickened. In the later stages, the red cells—those within the capillaries as well as those forming part of the alveolar exudate—appeared to fuse into agglomerated masses staining intensely with eosin, and such fused masses of erythrocytes formed capillary thrombi and entered into the matrix of the alveolar exudate. In the latter stages of the lesions, the derivation of this pink-staining material in which were embedded the fragmented leucocytes, was not apparent, but by following successive phases, it seemed clear that the hemoglobin

masses from the fusion of red blood corpuscles were the source of this material. The usual picture of hemolysis, with the preservation of the shadows of the hemolyzed cells, was never seen; nor was the picture that of rhexis.

The leucocytes of the exudate in the earlier stages were swollen and hydropic—often with a distinct cell membrane and a watery clear cytoplasm. Later the nucleus underwent fragmentation. The fibrin, delicate at first, later became swollen and the threads merged into larger clumps and masses.

The most striking and interesting feature of these experimental lesions, however, was the unrestrained growth of the pneumococci. In all the animals of this group, enormous numbers of diplococci were present, in many alveoli forming dense colonial masses. In sections stained by the Gram-Weigert method, the bacteria were so numerous as to impart a blue color to the section. They were often present in abundance in the edematous tissue about the blood vessels and the bronchi, as well as in the alveolar exudate.

3. Guinea Pigs Which Survived 7 and 14 Days Respectively after the Intratracheal Injection of Pneumococcus Culture and Toxic Autolysates.

The guinea pig which died in 7 days was slightly dyspneic after the first day and continued in this condition until death. At autopsy, the lungs were collapsed but appeared otherwise normal except for the left lower lobe which was slightly consolidated and of a translucent grayish color. Cultures from the heart and left lower lobe of this guinea pig gave a profuse growth of pneumococci. Histologically, the section through the left lower lobe showed the capillaries to contain little blood; the endothelial nuclei were swollen and hyperchromatic, the alveolar epithelial cells large and vacuolated. In the connective tissue about the main bronchus were several circumscribed masses of polymorphonuclear cells—the only evidence of an acute inflammatory reaction. The edematous peribronchial tissue was becoming organized by the ingrowth of fibroblasts. The lesions indicated recovery from a previous pneumonia.

Guinea Pig 84 died on the 14th day, having shown no signs of illness at any time. No gross or microscopic lesions were found to indicate

the occurrence of a previous pneumonia. Either the animal was exceptionally resistant to the autolysate and pneumococci or there had been a technical error in the inoculation.

(*C*) *Results from the Intratracheal Injection of Pneumococcus Cultures and Broth.*

Ten guinea pigs were injected with mixtures of pneumococcus cultures and broth. None of these became dyspneic at any time; only three of them died, two on the 5th day and one on the 7th. At autopsy, the gross appearance of the lungs of these three was normal. Cultures from the heart blood yielded a profuse growth of pneumococci.

Histological Findings.—Ten guinea pigs were studied histologically, the three which died, described above, and seven additional guinea pigs which were killed at intervals of 1 to 4 days after the inoculation of varying amounts of culture. Lesions were found only in the animals which were killed during the first 48 hours. They consisted of a sparse emigration of polymorphonuclear leucocytes into a few alveoli only, accompanied by occasional red blood corpuscles. There was no fibrinous exudation. Indeed there was no massive consolidation comparable to that regularly occurring in the animals of Group B already described. Pneumococci were found in the sections of but two of this series—6-28, killed after 27 hours and 6-39, killed after 2 days—and not in excessive numbers. Both of these animals received a very much larger dose of the microorganism than was given to the animals of Group B, which received toxic autolysates in addition to the living pneumococci.

The three guinea pigs dying on the 5th and 7th days showed no pulmonary lesions whatsoever in spite of the general septicemia.

(*D*) *Results from the Intratracheal Iniections of Toxin and Broth.*

Ten guinea pigs were injected with toxin and broth. Of these, three pigs died; two died 2 hours after the injection, and one died 48 hours after the injection with characteristic symptoms and pathology of a guinea pig injected with 0.2 cc. of a strong toxin, described above, *viz.*, in the two pigs which died in 2 hours the lungs were patchy deep red and pink throughout; in the pig which died in 48 hours $\frac{2}{3}$ of the

lungs was consolidated and deep red. The cultures of the lungs and heart of these three pigs were sterile. The other seven guinea pigs in this series were more or less dyspneic after the inoculation but had practically recovered from this sickness by the next day and survived.

DISCUSSION.

If we contrast the symptoms and pathology in these three groups, it is evident that there are striking and significant differences in the reaction of the animal. In agreement with previous workers it has been impossible to produce extensive pneumonic lesions by the injection of living pneumococci alone, even in large doses. The lesions which followed the intratracheal administration of the living organism appeared early and were mild and transitory in character even when the doses were greatly in excess of those used in the other groups. The animals which succumbed died of septicemia without lung involvement.

In striking contrast to this behavior was the reaction of the animals which received in addition to the living pneumococci, a small dose of the toxic autolysate. A certain proportion of these died within a few hours with shock-like symptoms; and from our observation upon the effect of toxic autolysate alone it would seem that the poisonous effect of this substance was primarily responsible since death occurred before there had been opportunity for the multiplication of the bacteria. The changes in the lung may be summarized as an intense hemorrhagic edema with very early inflammatory reaction.

In animals surviving this first shock there developed regularly massive areas of consolidation often of the lobar type and accompanied by an amazing multiplication of the pneumococci throughout the lung. The growth of the bacteria seemed indeed to outstrip the inflammatory reaction, since one found them even in areas outside of the pneumonic zones. It is clear that the toxic substances have in some way blocked the defensive mechanism and made conditions favorable for unrestrained growth. We shall not at this time attempt to analyze this further, or speculate as to the factors responsible for this striking phenomenon.

Experiments also demonstrated the marked toxicity of the bacterial autolysates prepared after the described manner. The lesions pro-

duced were intense and widespread in the lungs. The chief differences noted histologically between the reactions to the toxic autolysates alone, and the reaction when the poisonous substance was accompanied by the introduction of the living organism, are that there is less fibrin in the former group and the exudate appears to be somewhat looser in character. The alteration of the red blood corpuscles which was a striking feature in the presence of the living bacteria, was not seen with the toxic autolysate alone. Furthermore, as one might expect, there is an attempt at recovery and repair on the part of the animals which have been allowed to live for 3 days or more. These observations upon the primary toxicity of the autolysates brings up, of course, the interesting problem as to the rôle of similar substances formed *in vivo*, in the production of pneumonia in man.

CONCLUSIONS.

1. Anaerobic autolysates of pneumococci, prepared according to the method described, are highly toxic for guinea pigs when injected intratracheally in dosage of 0.2 cc. Death occurs either within a few hours (36 per cent) or within 3 days. In the early deaths there is intense hemorrhagic edema of the lungs with beginning inflammatory reaction; in animals surviving for 18 hours or longer extensive areas of pneumonia are produced.

2. The intratracheal injection of virulent living pneumococci is followed by transient slight lesion with recovery, or by later death from septicemia without pneumonic lesions.

3. The addition of a sublethal dose of toxic autolysate to living pneumococci alters the reaction of the animal, so that there develops extensive pneumonia associated with unrestrained multiplication of the organism.

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EXPLANATION OF PLATES.

PLATE 18.

FIG. 1. G. P. 8-49. Killed 29 hours after intratracheal injection of toxic autolysate alone. Patchy areas of pneumonia and diffuse edema.

FIG. 2. G. P. 8-53. Killed 24 hours after intratracheal injection of pneumococci alone. Sparse emigration of polymorphonuclears into a few alveoli. No pneumonic consolidation.

PLATE 19.

FIG. 3. G. P. 10-29. Killed 48 hours after intratracheal injection of pneumococci plus toxic autolysate. Diffuse pneumonic consolidation, with abundant growth of pneumococci.

FIG. 4. G. P. 6-27. Died 24 hours after intratracheal injection of toxic autolysate and pneumococci. Unrestrained growth of pneumococci in lung. (Gram-Weigert stain.)

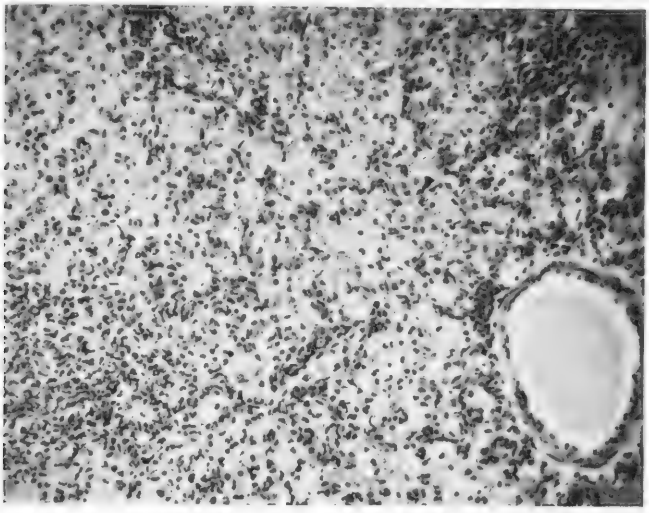

FIG. 1.

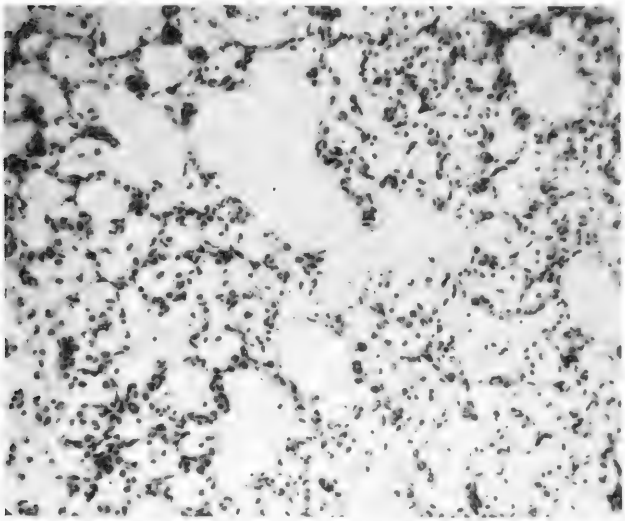

FIG. 2.

(Parker and Pappenheimer: Pneumonia in guinea pigs. I.)

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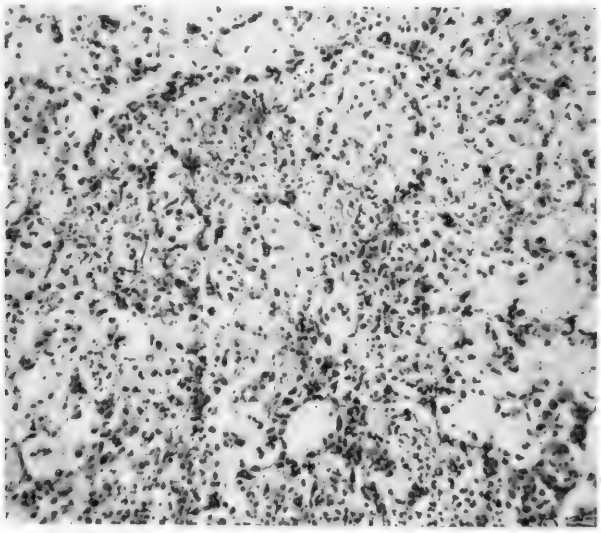

FIG. 3.

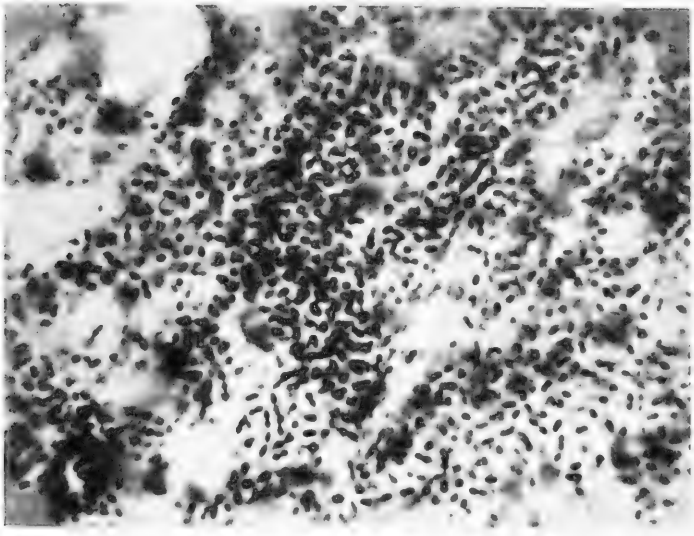

FIG. 4.

(Parker and Pappenheimer: Pneumonia in guinea pigs. I.)

STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

XI. An Inquiry into the Mode of Action of Antibacteriophage Serum.

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That sera of animals immunized by parenteral introduction of lytic filtrates have the property of neutralizing bacteriophage has been recognized since the work of Bordet and Ciuca in 1921 (1), but this demonstration of a new antigen was at the time of purely academic interest. In 1925, however, Sonnenschein (2), and shortly afterward Katsu (3), stated that human blood possessed antibacteriophagic properties in relation to certain infectious diseases, and they suggested that the reaction might be of diagnostic value. This possibility has made the serological study of bacteriophage of considerable practical importance.

Methods.

Purification of Phages.—It is a common observation that when a lytic filtrate is first obtained it ordinarily contains a number of different principles, or bacteriophages. The first procedure in the purification of this mixture is to carry lysis in series through a number of passages on the bacterial strain, thus eliminating by dilution any phages that act only on heterologous bacteria.

It is probable that even the filtrate thus obtained still consists of several different components which act on the same bacterial species, and which can be further separated by the plating method. For each phage forms a characteristic plaque, as has been demonstrated by Bail (4) and by Gratia (5). Plaques of the same sort should be picked for several passages, in this manner securing a pure phage.

Pure phages obtained in this manner were used as antigens. Immunization was conducted by intravenous and subcutaneous injections, and in both cases neutralizing sera were obtained. The sera of mice, on the other hand, that were fed phage daily for 6 weeks, were not capable of neutralizing the phage ingested.

Technique for Demonstrating Neutralization.—Bordet and Ciuca (1) demonstrated neutralization by adding a mixture of a specific serum and the appropriate phage to a culture of susceptible bacteria in broth, the absence of visible lysis serving as a criterion of neutralization. They also demonstrated (6) in a control experiment, that normal serum did not prevent phage action. D'Herelle and Eliava (7), using this technique, found that on prolonged incubation lysis took place, and so concluded that the serum merely inhibited the lysis, but that it did not inactivate the phage. Wagemans (8), however, found that if the time of contact between phage and serum was sufficiently long, neutralization was complete and the phage could not be regenerated. A technique commonly employed (9) has been to mix phage and serum for a varying length of time and then to streak this mixture on a plate seeded with susceptible bacteria, neutralization

TABLE I.

The Neutralization of Coliphage by Immune Serum.

Phage	Antigen of serum	Bacterium	Phage dilution				
			Undiluted		1–100	1–10,000	1–1,000,000
			Titer	Slant	Slant	Slant	Slant
P.C.	Normal serum	*B. coli*	10^{-9}	+++	++	100±	−
	P.C. coliphage	" "	10^{-4}	−	−	−	−

+++ indicates complete lysis.

++, +, indicate lesser degrees of lysis.

Numbers indicate the number of plaques.

± following a number indicates that the number of plaques was estimated.

− indicates absence of lysis.

These signs are used in all of the following protocols in which lysis on agar slants is recorded.

being demonstrable by the absence of plaque formation. Sonnenschein (10) did this in a quantitative manner, mixing diluted phage with undiluted serum on the one hand, and undiluted phage with diluted serum on the other, running drops of the mixtures down the previously inoculated surface of an agar plate and observing lysis after incubation.

In our search for a satisfactory technique the findings of Wagemans (8) were first repeated and confirmed. It was found, however, that the time required for complete neutralization depends on the potency of the serum, a serum of high potency causing complete neutralization in a comparatively short time. Inhibition of lysis in broth proved unsatisfactory as a method, since a partial neutralization can delay or prevent visible lysis, although increase of the phage may proceed at a fairly rapid rate. For detecting partial neutralization it has been found

TABLE II.

The Specificity of Antiphage Sera.

Phage	Antigen of serum	Bacterium	Dilution of phage				
			Undiluted		1-100	1-10,000	1-1,000,000
			Titer	Slant	Slant	Slant	Slant
P.C.	Normal	*B. coli*	10^{-9}	+++	++	+	14
	P.C. phage		10^{-5}	−	−	−	−
	Laudman phage		10^{-9}	+++	++	+	11
	P. I. D. "		10^{-10}	+++	++	+	5
	B.H. "		10^{-10}	+++	++	+	8
	Normal	*B. dysenteriæ* Shiga	10^{-9}	+++	+++	+	15
	P.C. phage		10^{-6}	−	−	−	−
	Laudman phage		10^{-9}	+++	+++	+	20
	P.I.D. "		10^{-9}	+++	+++	+	25
	B.H. "		10^{-9}	+++	++	+	6
Laudman	Normal	*B. coli*	10^{-10}	+++	++	+	3
	P.C. phage		10^{-9}	+++	++	+	4
	Laudman phage		10^{-3}	−	−	−	−
	P.I.D. "		10^{-10}	+++	++	+	−
	B.H. "		10^{-9}	+++	++	+	3
	Normal	*B. dysenteriæ* Shiga	10^{-9}	+++	+++	++	16
	P.C. phage		10^{-10}	+++	+++	++	7
	Laudman phage		10^{-5}	−	−	−	−
	P.I.D. "		10^{-10}	+++	+++	++	8
	B.H. "		10^{-9}	+++	+++	++	−
P.I.D.	Normal	M.T.[2]	10^{-8}	+++	++	+	12
	P.C. phage		10^{-8}	+++	++	+	−
	Laudman phage		10^{-8}	+++	++	+	7
	P.I.D. "		10^{-3}	−	−	−	−
	B.H. "		10^{-7}	+++	++	+	10
B.H.	Normal		10^{-8}	+++	++	100±	1
	P.C. phage		10^{-8}	+++	++	100±	−
	Laudman phage		10^{-9}	+++	++	100±	−
	P.I.D. "		10^{-8}	+++	++	100±	3
	B.H. "		10^{-3}	−	−	−	−

convenient to drop a mixture of phage and antiserum on an evenly inoculated agar surface, and to compare the resulting lysis with that caused by a mixture of phage and normal serum. Absence of lysis under these conditions, however, does not necessarily indicate complete inactivation of the phage.

As the reaction of neutralization may at times continue for several days, it was found best to limit the time of contact of phage and serum to a fixed period and then to determine quantitatively, by the titration method of Appelmans (11), the amount of phage remaining active. In addition to this, serum was mixed with serial dilutions of the phage, and after the given period of contact a drop of each

TABLE III.

The Specificity of Neutralization of Adapted Phages.

Phage	Antigen of serum	Susceptible bacterium	Phage dilution				
			Undiluted		1-100	1-10,000	1-1,000,000
			Titer	Slant	Slant	Slant	Slant
P.C. phage adapted to Shiga bacillus	Normal	*B. coli*	10^{-8}	+++	++	+	2
	P.C. phage		10^{-3}	−	−	−	−
	Laudman phage		10^{-8}	+++	++	+	11
	Normal	*B. dysenteriæ* Shiga	10^{-8}	+++	++	100±	2
	P.C. phage		10^{-3}	−	−	−	−
	Laudman phage		10^{-8}	+++	++	100±	1
Laudman phage adapted to *B. coli*	Normal	*B. coli*	10^{-10}	+++	+++	150±	5
	P.C. phage		10^{-9}	+++	+++	150±	5
	Laudman phage		10^{-3}	−	−	−	−
	Normal	*B. dysenteriæ* Shiga	10^{-10}	+++	+++	+	14
	P.C. phage		10^{-10}	+++	+++	+	20
	Laudman phage		10^{-4}	−	−	−	−

of these mixtures was run down the surface of agar slants previously inoculated with the susceptible bacterium. The results with immune serum were then compared with those with normal serum. A typical protocol is given in Table I, which shows the neutralization of P.C. coliphage by its specific antiserum.

EXPERIMENTAL.

Specifiicity of Neutralization.—Maisin (12), in 1921, stated that antilytic sera are not specific in their action, but this finding lacks confirmation. In most of the experiments here to be reported four bacteriophages were used. They are listed below and their respective activity is indicated.

1. P.C. coliphage, acting on *B. coli* and *B. dysenteriæ* Shiga.
2. Laudman Shiga phage, acting on *B. coli* and *B. dysenteriæ* Shiga.
3. P.I.D. M.T.[2] phage, acting on M.T.[2] (*B. pestis caviæ*).
4. B.H. staphylococcus phage, acting on *Staph. aureus* "G."

In addition to these, B.W. coliphage, acting on *B. coli*, was used in some experiments. Neutralizing sera were prepared for each of the phages, and then the ability of each antiserum to neutralize each of the phages was determined. The result of these studies is given in Table

TABLE IV.

Specificity of Antisera for Different Phages Acting on the Same Bacterium.

Phage	Antigen of serum	Bacterium	Phage dilution				
			Undiluted		1–100	1–10,000	1–1,000,000
			Titer	Slant	Slant	Slant	Slant
P.C. coliphage	Normal	*B. coli*	10^{-9}	+++	++	+	14
	P.C. phage	" "	10^{-5}	–	–	–	–
	Laudman phage	" "	10^{-9}	+++	++	+	11
	B.W. "	" "	10^{-9}	+++	++	+	12
Laudman coliphage	Normal	" "	10^{-10}	+++	+++	150±	5
	P.C. phage	" "	10^{-9}	+++	+++	150±	5
	Laudman phage	" "	10^{-4}	–	–	–	–
	B.W. "	" "	10^{-9}	+++	+++	150±	10
B.W. coliphage	Normal	" "	10^{-7}	+++	++	4	–
	P.C. phage	" "	10^{-7}	+++	++	2	–
	Laudman phage	" "	10^{-6}	+++	++	3	1
	B.W. "	" "	10^{-4}	1	–	–	–

II, in which it is seen that each serum exerts a specific neutralizing action on its own antigen, though it is without influence on the other phages.

The specificity of neutralization after the phage had been adapted to another bacterium was next studied. Seiffert (13) found neutralization to be specific after adaptation. Wagemans (8), Wolff and Jansen (14), and Kasarnowsky and Tiomkin-Schukoff (15), on the other hand, found that while specific neutralization usually occurred, this was not invariably the case. The results of our inquiry into this

TABLE V.

The Effect of Antibacterial Sera on Phage.

Phage	Antigen of serum	Bacterium	Phage dilution				
			Undiluted		1–100	1–10,000	1–1,000,000
			Titer	Slant	Slant	Slant	Slant
P.C. coliphage	Normal	*B. coli*	10^{-10}	+++	++	+	6
	B. coli	" "	10^{-9}	+++	++	+	1
	" " autolysate	" "	10^{-9}	+++	++	+	8
Laudman Shiga phage	Normal	" *dysenteriæ* Shiga	10^{-9}	+++	+++	++	12
	B. dysenteriæ Shiga	" " "	10^{-9}	+++	+++	++	10
	" " " autolysate	" " "	10^{-9}	+++	+++	++	6
P.I.D. M.T.[2] phage	Normal	M.T.[2]	10^{-7}	++	+	20	–
	M.T.[2]	"	10^{-7}	++	+	20	–
	" autolysate	"	10^{-7}	++	+	15	–
B.H. staphylococcus phage	Normal	*Staph. aureus* G	10^{-7}	+++	++	100±	–
	Staphylococcus G	" " "	10^{-6}	+++	++	100±	–
	" " autolysate	" " "	10^{-6}	+++	++	100±	–

question are given in Table III, and show that, with the material at our disposal, neutralization was specific for the original phage, even though it may have been carried for a number of passages on another bacterial species. The use of imperfectly purified phages may explain some of the discrepancies in the findings quoted, but the possibility that the antigenic properties of a phage may alter with adaptation to another bacterial species cannot be excluded as a property of some phages.

The specificity of the neutralizing power of sera prepared against different phages acting on the same bacterium was also studied. Bruynoghe and Appelmans (16), and Bail and Watanabe (17) described two serologically distinct phages acting on the same bacterial

TABLE VI.

Agglutination of Bacteria by Antiphage Sera.

Serum	Bacterium	1-10	1-20	1-40	1-80	1-160	1-320	1-640	1-1280	1-2560	1-5120	1-10,240	Control
Anti P.C. coliphage	*B. coli*	−	−	−	−	−	−	−	−	−	−	−	−
" Laudman Shiga phage	*B. dysenteriæ* Shiga	−	−	−	−	−	−	−	−	−	−	−	−
Anti P.I.D. M.T.[2] phage	M.T.[2]	+++	+++	+++	++	++	+	+	+	+	±	−	−
Anti B.H. staphylococcus phage	Staphylococcus	+++	+++	+	+	+	±	−	−	−	−	−	−

species. A later observation of interest is that of Watanabe (9) who found two phages, acting on the same bacterial species, that were similar serologically but distinguishable by other means. In our experiments P.C., Laudman, and B.W. phages, all acting on *B. coli*, were used, and the serum for each was found to be specific for its own antigen and without effect on the other two phages (Table IV).

The Effect of Antibacterial Sera on Phage.—Bail (18), in 1921, found that antibacterial sera neutralized phage, but that they were only one hundredth as strong as homologous antilytic sera. This finding has not been confirmed in other laboratories. That antibacterial sera in sufficient concentration can inhibit lysis in broth has been recognized (19), but this has been shown (20) to be due to a direct

action of the serum on the bacteria, and not to a process of neutralization of phage.

We have investigated the question thus brought up, and have used not only agglutinating sera, but also precipitating sera prepared against autolyzed broth cultures of bacteria. The results are given in Table V, which shows that no neutralizing antibodies were present in the sera.

However, when lysogenic cultures were used as antigens the antisera obtained exhibited definite antilytic properties. This experiment was carried out as follows:

Bacteria were made artificially lysogenic by continuing incubation after lysis in broth, and inoculating the overgrowth of resistants directly onto agar slants. When animals were immunized with these cultures, their serum, in addition to its agglutinating properties, became capable of neutralizing the phage carried; but here, of course, phage was also present in the antigen.

Presence of Other Antibodies in Antiphage Sera.—Since the filtrates used as antigens contained, in addition to the active bacteriophage, bacterial protein liberated during growth and subsequent lysis, the appearance of antibacterial antibodies was to be expected. These appeared with regularity, and antilytic sera prepared by immunization of animals with crude filtrates of lysed cultures regularly formed a precipitate when mixed with autolysates of the homologous bacterium, or with the filtrate used as antigen. Bruynoghe and Dubois (21) have found that precipitation is to some extent specific for the bacteriophage. In our hands the method has been unsatisfactory, since with the presence of degradation products of bacteria of closely related species there is enough non-specific precipitation to obscure the results.

The presence of agglutinins in antilytic sera is usual (22, 23). Marshall (24), however, prepared a serum that did not agglutinate the homologous bacterium. The results of our studies have been variable. From Table VI it will be seen that sera against the coliphage and the Shiga phage did not agglutinate the homologous bacteria, while those against M.T.[2] phage and staphylococcus phage were definitely agglutinating. In the case of staphylococcus agglutination occurred with normal sera also, so that the results are not so striking as the truly specific reactions obtained with M.T.[2]

Complement fixation was studied by Sanderson (25) and later by Flu (26). Their findings showed that with rabbit serum complement is fixed non-specifically in the presence of bacterial protein and the reaction is not related to the presence of bacteriophage; occasionally complement may be fixed even with plain broth. In view of these reports complement fixation was not tested by us.

Independence of the Two Types of Antibodies.—That the antibodies against the bacteria and those against the phage are distinct can be readily demonstrated by the absorption test. The antibodies against the bacteria may be absorbed, leaving unreduced the antilytic power of the serum. This fact has been observed in several laboratories (23, 27). It is also possible to remove a precipitate resulting from a mixture of a bacterial autolysate and antilytic serum without reducing the neutralization titer of the serum.

The Reaction of Neutralization.—In order to get some conception of the nature of the reaction when bacteriophage is neutralized by antiserum, a study of the factors entering into the reaction was made.

(*a*) *Rate of Neutralization.*—When phage and antiserum are placed in contact with each other, neutralization proceeds at first rather rapidly, but the rate of this process gradually decreases. Seisser (28) found that the greater part of the phage is neutralized within 24 hours, and Wagemans (8) found that complete neutralization may require as long as 4 days. Higher temperatures, according to Otto and Munter (29), increase the rate of neutralization. This was confirmed by Arnold and Weiss (30).

All of these findings have been confirmed in our laboratory.

Arnold and Weiss found that neutralization follows the law of multiple proportions and also reported the Danysz-Bordet phenomenon (30).

(*b*) *The Rôle of Complement.*—Osumi (31) found complement necessary for neutralization of bacteriophage by immune serum, but this finding has not been confirmed by other workers, and in our experiments complement played no part in the reaction.

(*c*) *Mode of Neutralization.*—Seiffert (13) reported that neutralization proceeds by a reduction in size, rather than number, of the plaques. Grigorieff (32), on the contrary, found that serum acts by inactivating individual units. With the material used in the present

work the latter has been found to be the mode of action. Reduction in size of the plaques has not been observed.

(*d*) *Nature of the Reacting Substances.*—That the action of the serum is directly on the bacteriophage and not on the bacteria or bacterial products has been demonstrated in the preceding pages by the specificity of their neutralization, by the failure of antibacterial sera to affect them, and by the undiminished antilytic activity of neutralizing sera after the complete removal of antibacterial antibodies.

(*e*) *The Influence of Adsorption.*—There is a possibility that phage may be inactivated by adsorption onto a precipitate which is formed when antiserum is combined with a filtrate containing bacterial protein. This possibility was tested by means of attempts to remove phage from a filtrate by the formation of a heterologous precipitate. Horse serum and homologous immune rabbit serum were mixed in the presence of phage and incubated overnight. The tube was then centrifuged and the clear supernatant fluid titrated. The lytic activity was found equal to that of another sample of phage similarly diluted with normal serum. Moreover, precipitation of filtrates by antibacterial sera failed to reduce the titer of phage. For these reasons it seems unlikely that adsorption can be the explanation of neutralization.

(*f*) *Reversibility of the Neutralization.*—The view that the neutralization of phage is analogous to the neutralization of toxin by antitoxin has been offered by certain workers who present evidence that the combination can dissociate. Otto and Munter (29) reported that dilution dissociated the combination but their method does not exclude the possibility of incomplete neutralization as the explanation. Weiss (33) has reported the complete dissociation of a neutral mixture by tryptic digestion.

We have attempted, though without success, to effect dissociation (1) by changing the reaction, and (2) by digestion with trypsin.

1. Dissociation by Changing the Reaction.—After determining the activity of a serum it was mixed with phage in the proportion to secure a neutral mixture (in this case 1–10). The resulting inactive fluid was diluted 1–5 in buffers of various degrees of acidity or alkalinity and these mixtures were left at room temperature overnight, when they were tested for phage. Phage alone, and serum alone similarly diluted and treated in an identical manner, were found to be unaffected. Table VII shows that dissociation was not secured by this method.

2. Dissociation by Digestion with Trypsin.—Trypsin solution was prepared by suspending 2 per cent of Fairchild's trypsin in borax-boric acid buffer at pH 7.4, and placing the flask in the ice box overnight. This material was then filtered through a Berkefeld V candle, and the reaction adjusted colorimetrically with

TABLE VII.

Attempt to Dissociate a Neutral Phage-Antiphage Mixture by Changing the Reaction.

pH of buffer	4.05	4.99	5.94	6.43	7.03	7.54	7.96	8.97	9.90
Quantity of buffer, *cc.*	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Quantity of neutral mixture, *cc.*	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Mixtures left at room temperature overnight									
Titer	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

TABLE VIII.

Effect of Trypsin on Neutral Phage-Antiphage Mixture.

Day of incubation	Immediate	1	2	4	5	6
Phage with normal serum	10^{-8}	10^{-6}	10^{-8}	10^{-6}	10^{-7}	10^{-6}
" " immune "	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

TABLE IX.

Effect of Trypsin on Antiphage Serum.

Serum	Undiluted		1-100	1-10,000	1-1,000,000
	Titer	Slant	Slant	Slant	Slant
Normal	10^{-9}	+++	++	150±	4
Immune before digestion	Neg.	−	−	−	−
Digested 1 day	10^{-6}	+	25±	−	−
" 2 days	10^{-7}	++	100±	1	−
" 4 "	10^{-7}	++	150±	1	−
" 6 "	10^{-7}	++	100±	−	−

sterile sodium hydroxide to a pH of 7.4. One volume of this solution was added to three volumes of a neutral mixture of coliphage with the corresponding antiserum. Phage with normal serum was similarly treated as a control, and the two tubes were placed in the incubator at 37°C. The solution of trypsin was shown

to be active in a duplicate tube to which a Mette tube was added. The activity of the phage in each of the two tubes was determined at intervals (Table VIII). At the same time, immune serum was similarly digested with trypsin and its ability afterwards to neutralize phage was determined at the same intervals (Table IX).

The neutralizing power of the serum alone was markedly diminished by the trypsin, but, in spite of this, the phage was not liberated from a neutral mixture with this same serum by the action of trypsin. These experiments were repeated with other phages and antisera, but no definite evidence of dissociation was obtained.

That neutral mixtures may dissociate under certain conditions is suggested, however, by the fact that when rabbits are immunized with a neutral mixture of phage and immune rabbit serum, their serum becomes capable of neutralizing phage.

Stability of the Bacteriophage Antigen.—Arnold and Weiss (34), and Asheshov (35) have shown that phage inactivated by heat is still antigenic. We have confirmed this. However, boiling, or autoclaving at 120°C. for 1 hour renders the phage antigenically inert, as determined by the usual course of immunization. After inactivation of phage by formalin, by immune serum, or by adsorption onto dead bacteria, its antigenic property remains unchanged.

The Occurrence of Neutralizing Antibodies in Disease.—As stated previously, Sonnenschein (2) and Katsu (3) detected antilytic properties of human sera and related these findings to specific infections. The subject has been studied experimentally in this laboratory by feeding the bacteria of mouse typhoid (M.T.[2]) to mice and testing the serum of those animals surviving the infection for neutralizing antibodies. Three phages acting on this bacterium were available, and none of these was neutralized by the serum of the surviving mice. Moreover, the spontaneous appearance of neutralizing antibodies in experimental animals has never been observed in this laboratory.

DISCUSSION.

The reaction between bacteriophage and specific neutralizing antiserum constitutes a definite serological phenomenon, different from and independent of any of the known reactions between bacteria or bacterial products and their antisera. Each bacteriophage is a specific antigen independent from the bacterial antigens present in lytic

filtrates, and the antigenic properties are affected by the same influences that affect other known antigens, as for example heat. The reaction of neutralization seems to be analogous to the neutralization of toxin by antitoxin, in that complement is unnecessary, neutralization is not instantaneous but takes some time, and the reaction follows the law of multiple proportions. The fact that each phage is neutralized only by its specific antiserum makes possible the serological identification and classification of bacteriophages.

Antilytic sera may at times be of some value in the laboratory. A mixture of phages may be partially purified by using a serum which neutralizes one or more components, leaving the remainder active and thus simplifying the problem of their separation. Lysogenic cultures may be rendered free of phage by cultivation in broth containing serum active against the phage carried. The ensuing neutralization of phage permits the culture to return to its original state.

The use of the reaction of neutralization in the diagnosis of disease seems, in view of the results reported here, to be impractical. The failure of antibacterial sera to neutralize phage, and the indefinite number of phages acting on the same bacterial species would render the test uncertain. Furthermore, only positive results would be of any significance, since it would be difficult to exclude the presence of antibodies against some other bacteriophage acting on the same bacterium.

SUMMARY.

1. Each bacteriophage is a specific antigen.

2. The antibodies against the bacteriophage are independent of those against the bacterial substrate used in preparing the phage.

3. The reaction of neutralization is closely analogous to that of the neutralization of toxin by antitoxin.

4. The serum of mice experimentally infected with mouse typhoid (M.T.[2]) did not become capable of neutralizing phages acting on the infecting organism.

5. The use of the reaction of neutralization of bacteriophage in the diagnosis of disease as proposed by Sonnenschein seems impractical.

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STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

XII. Concerning the Production of Phage from Bacterial Cultures.

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The development of bacteriophage in bacterial cultures has been described repeatedly. The importance of the phenomenon is self-evident. If the production of phage from bacteria themselves can be incontrovertibly proved, then it is scarcely conceivable that the bacteriophage can be a living ultramicroscopic parasite of the bacteria.

In some cases, plaques from which lysis in series can be conducted have appeared spontaneously in stock cultures (1). In most cases, however, a more complicated procedure has been necessary (2) for the development of the phage, one involving repeated filtrations and reinoculations, sometimes of young cultures, but usually of cultures that have aged or been treated with some chemical or physical agent. The objection to all of the results is that they cannot be reproduced at will, and although positive results may predominate in the hands of some workers, the outcome of individual experiments is uncertain. Furthermore, the amount of manipulation necessary increases the possibility of contamination with phage, and this factor becomes of increasing importance when one considers the ubiquity of phage and its relative resistance to most chemical and physical agents. Consistent results were reported at one time by Putter and Vallen (3), but they subsequently traced them to the use of contaminated filters (4), and therefore retracted their previous statements. The possibility that bacteriophage was already present in the cultures used must also be considered. In this connection Manninger (5) takes the extreme view that all of the cultures of the colon-typhoid-paratyphoid group of bacteria are contaminated with bacteriophage.

A phenomenon distinct from these, however, and one which can be

regularly brought about is the production of phage for one bacterium in cultures of another.

This was first described in 1922 by Lisbonne and Carrère (6), and they considered it the result of bacterial antagonism. Kuttner (7), in 1923, produced phage from stock cultures, and the phage produced acted only on heterologous strains, and not on the strain from which it was derived. In 1924, Gildemeister and Herzberg (8) described a bacterium (*Coli* 88) having the property, like the *B. coli* strain of Lisbonne and Carrère, of initiating serial lysis of the Shiga bacillus. These two bacterial strains have similar, if not identical, properties, so the literature on the two will be summarized together.

Bordet was able, by culturing from single colonies, to secure strains that would not produce phage for the Shiga bacillus (9), and by subculturing single colonies for a number of passages, he could get strains that were to some extent lysogenic for each other (10). Since the great majority of the strains from single colonies could still produce phage for the Shiga bacillus, he called this phenomenon "active lysogenesis," as contrasted to "passive lysogenesis" which resulted from artificially mixing a culture with phage, and in which the phage was only perpetuated in mass cultures.

D'Herelle (11) repeated Bordet's experiments, and found that in artificially produced lysogenic cultures phage persisted in transfers from single colonies if the "rough" colonies were selected. He thought that such colonies indicated symbiosis of the bacteriophage with the bacteria. Bail (12) confirmed d'Herelle's experiments and agreed with him that the phenomenon did not represent a true production of phage from the bacteria themselves but resulted from the admixture of phage with the bacteria. McKinley (13) immunized animals with broth cultures of the *coli* strain of Lisbonne and Carrére and the resulting sera neutralized the phage already produced by the culture, but would not render the strain of bacteria non-lysogenic.

To explain the phenomenon of phage production for the Shiga bacillus in cultures of *B. coli* on the basis of phage preexisting in the culture, it is necessary to assume that the colon bacillus employed for the work carries a phage active against both *B. coli* and against *B. dysenteriæ* Shiga, and that the particular strain of *B. coli* employed is resistent to this phage to such an extent that no gross lysis occurs, although enough individuals in the culture prove susceptible to serve for the perpetuation of the phage. It should be possible to reproduce the phenomenon experimentally, and this we have attempted to do in the following work. In addition to using a phage acting on both *B. coli* and *B. dysenteriæ* Shiga, a monovalent phage, acting only on *B. coli*, was used. In the case of this latter the original strain of bacteria was necessary to demonstrate the presence of phage.

EXPERIMENTAL.

The Production of Lysogenic Bacteria.—*B. coli*, and P.C. phage, which acts both on *B. coli* and on *B. dysenteriæ* Shiga, were inoculated into a tube of broth, and incubation was continued after the completion of lysis. The resulting overgrowth was inoculated directly onto agar slants. The growth appeared normal, but phage was readily demonstrated by cultivating in broth either with *B. dysenteriæ* Shiga or with the original strain of *B. coli*, then removing the living resistant bacteria by filtration, centrifugation, or heating to 56°C. for 40 minutes, and adding some

TABLE I.

Serological Identification of Phage Recovered from Lysogenic Bacteria.

Phage derived from:	Serum	Bacterium	Phage dilution			
			Undiluted	1–100	1–10,000	1–1,000,000
P.C. lysogenic *B. coli*	Normal	*B. coli*	+++	++	100±	−
	Anti P.C. phage	" "	−	−	−	−
	" B.W. "	" "	+++	++	100±	−
B.W. lysogenic *B. coli*	Normal	" "	+++	+++	26	1
	Anti P.C. phage	" "	+++	+++	23	1
	" B.W. "	" "	++	++	−	−

+++ = complete lysis.
++, + = lesser degrees of lysis.
Numbers indicate the number of plaques.
± following a number indicates that the number was estimated.
− indicates absence of lysis.
These signs will be used in the following protocols whenever lysis on agar slants is recorded.

of this material to a fresh culture of the susceptible organism. Phage could in this way be demonstrated even after the bacteria had been cultivated in series through 80 daily passages. A like experiment was done with B.W. coliphage, which acts only on *B. coli*. The phage could be demonstrated by allowing the resulting lysogenic strain to act on the original strain of colon bacilli.

Identification of the Phages Produced by Lysogenic Bacteria.—It was necessary to ascertain that the phages produced were those to which the bacteria were originally exposed. This was readily done, as the two phages are distinct serologically, and a serum prepared against one of the phages is inactive against the other.

Serial dilutions of the phages recovered were mixed with equal quantities of sera prepared against the original phages, and after an interval of 1 hour at room temperature, drops of the mixtures were run down the center of agar slants previously inoculated with the susceptible organism (Table I).

The phage recovered from the culture made lysogenic by exposure to P.C. phage was found to be neutralized by anti P.C. phage serum and not by anti B.W. phage serum, while the reverse was true of the phage recovered from the culture exposed to the action of B.W. phage. This establishes the identity of the phages recovered from the bacteria with those to which the cultures were originally exposed.

Serological Detection of Phage in Lysogenic Cultures.—It seemed reasonable to

TABLE II.

Phage Neutralization by Sera Prepared against Lysogenic Bacteria.

Phage	Serum	Bacterium	Phage dilution			
			Undiluted	1–100	1–10,000	1–1,000,000
P.C. coliphage	Normal	*B. coli*	+++	++	−	−
	Anti killed P.C. lysogenic	" "	−	−	−	−
	" live " "	" "	−	−	−	−
B.W. coliphage	Normal	" "	+++	++	60±	−
	Anti killed B.W. lysogenic	" "	++	40±	1	−
	Anti live B.W. lysogenic	" "	−	−	−	−

assume that if phage is present in a culture, it should act as an antigen, and consequently, an animal immunized against a lysogenic culture should give a serum capable of neutralizing the phage carried. Animals were therefore immunized by intravenous injection, with the following antigens:

1. P.C. lysogenic *B. coli*, 18 hour broth culture.
2. P.C. lysogenic *B. coli*, heat-killed saline suspension from an 18 hour agar slant culture.
3. B.W. lysogenic *B. coli*, 18 hour broth culture.
4. B.W. lysogenic *B. coli*, heat-killed saline suspension from an 18 hour agar slant culture.

The sera of the immunized animals were then tested for ability to neutralize P.C. and B.W. coliphages, and the results are recorded in Table II. From this it is seen that the sera of animals immunized with lysogenic cultures are capable

of neutralizing the phage carried. It has been shown in a previous communication (14) that antibacterial sera are without effect on phage, so the ability of an antibacterial serum to neutralize bacteriophage must indicate that the bacteriophage was present in the culture used as antigen.

An attempt was then made to carry out analogous experiments with a culture of colon bacilli that was lysogenic when isolated. A culture of the *B. coli* strain of Lisbonne and Carrère was kindly furnished us by Dr. E. B. McKinley, and a rabbit was immunized by intravenous injections of 18 hour broth cultures, freshly prepared for each injection. After the course of immunization usually followed in this laboratory (3 daily injections at weekly intervals for 4 weeks) the serum was tested for ability to neutralize the phage secured by the action of this strain of bacteria on the Shiga bacillus.

TABLE III.

Neutralization of Phage by Serum against B. coli Strain of Lisbonne and Carrère.

Phage	Serum	Bacterium	Phage dilution				
			Undiluted		1–100	1–10,000	1–1,000,000
			Titer	Slant	Slant	Slant	Slant
Phage from *coli* strain of Lisbonne and Carrère	Normal	Shiga L	10^{-8}	++	+	4	−
	Anti *B. coli* of Lisbonne and Carrère	" "	10^{-6}	+	30±	−	−

Table III shows that, while the degree of neutralization was not great, there was definite neutralization of the phage by the immune serum.

This is considered definite evidence that the phage was present in the culture. The antilytic property of serum of animals immunized against this bacterium has also been reported by McKinley (13) and confirmed by da Costa Cruz (15).

The phenomenon, as experimentally reproduced, consists in the demonstration of the phage which was previously mixed with the bacterial culture, and which remains present through an indefinite number of transfers.

DISCUSSION.

Consistent production of phage by one bacterium for another seems, in view of the experiments recorded here, to be the result of contamination of the "active" strain with a bacteriophage capable of acting on the susceptible strain. The majority of the individuals in the lysogenic strain are resistant and therefore such a culture cannot be used in the demonstration of the phage carried, unless, by picking a sufficiently large number of isolated colonies, a susceptible strain can be obtained. This has been done by Bordet, though his explanation does not agree with the one here given.

The phenomenon is readily reproducible experimentally by exposing a bacterial culture to the action of a polyvalent phage, the lysogenic strain thus obtained producing a phage that has the same range of activity as that possessed by the original phage. The apparent production of phage consists merely in the demonstration of the presence of the phage previously added, and that persists in the culture indefinitely. To demonstrate more conclusively that the phage recovered is the same one originally added to the culture, advantage can be taken of the fact that each phage is a specific antigen, and so the phage recovered can be identified with the original phage by its neutralization by specific antiserum.

Furthermore, the presence of phage in the bacterial culture may be detected by the immunization of animals, the resulting antibacterial serum being also capable of neutralizing the phage carried. As antibacterial sera are known to be incapable of neutralizing bacteriophage, the presence of neutralizing antibodies may be considered evidence that phage was present in the culture. The absence of neutralizing antibodies in an antibacterial serum, however, cannot be taken as positive evidence of the absence of bacteriophage, as antigens vary in their ability to stimulate antibody production. The possibility must be considered, therefore, that the phage carried may be a poor antigen and that no detectable antilytic properties may develop during the course of immunization. The development of antilytic properties during the immunization of animals with the *B. coli* strain of Lisbonne and Carrère shows definitely that phage was present in the culture.

The production of phage from bacterial cultures alone must be regarded as unproven, though this possibility must still be considered.

SUMMARY.

1. The phenomenon of phage production by one bacterial culture for another of different sort has been reproduced experimentally.

2. This phenomenon results from phage carried with the culture, and not from the spontaneous appearance of phage in a culture previously free from it.

3. Animals immunized against the lysogenic bacteria may develop antibodies that neutralize the phage carried.

4. The development of neutralizing antibodies on immunization with a bacterial culture is evidence of the presence of bacteriophage in the culture.

5. The failure of such antibodies to appear on immunization with bacteria does not necessarily indicate that bacteriophage is not present.

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ON THE INHERITANCE OF AGGLUTINOGENS OF HUMAN BLOOD DEMONSTRABLE BY IMMUNE AGGLUTININS.[1]

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Studies on the inheritance of serological properties were first undertaken systematically by von Dungern and Hirschfeld with the agglutinable substances in the blood of dogs (2) and with the human isoagglutinogens (3).[2] The authors named discovered the fact that the isoagglutinogens A and B are inherited as Mendelian dominants and this result has been amply confirmed by numerous workers.

According to their hypothesis there are two pairs of allelomorphic genes, *Aa*, and *Bb*, where *A* and *B*, the dominant genes, determine the presence of the corresponding agglutinogens, and *a* and *b*, the recessive genes, their absence. The genes for the blood groups are the following; group O:[3] aabb; group A: AAbb or Aabb; group B: aaBB or aaBb; group AB: AABB or AABb or AaBB or AaBb.

Another hypothesis has been advanced by Bernstein (6). He assumes multiple (three) allelomorphs, R, A, and B. The genetic formulæ accordingly are; group O: RR; group A: AA, AR; group B: BB, BR; group AB: AB. The theory of Bernstein does not involve a deviation from the older theory in the types of offspring except in the cases of parents belonging to group AB. According to the older view there may be children of any group in unions where one or both of the parents are in group AB; Bernstein's hypothesis, on the other hand, excludes children of groups O and AB in unions O × AB, and children O in unions A × AB, B × AB, or AB × AB. The recent work especially of Schiff (7), Thomsen (8), Preger (9), and Sievers (10) supports the opinion of Bernstein.[4, 5]

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[1] See the preliminary report (1).

[2] The problem of the inheritance of the human blood groups and a few results had been mentioned by Ottenberg and Epstein (4).

[3] The nomenclature of the blood groups by letters instead of numerals has been recommended both by the American Association of Immunologists and by the National Research Council (5) and is used in the present publication.

[4] The objection of Mendes-Correa (11) to the theory of Bernstein would imply

Tests for M and N in Several

Family No.	143						144					
Blood No.	F 298	M 299	300	301	302	303	F 304	M 305	306	307	308	
Group	A	A	A	A	A	A	O	O	O	O	O	
Reaction for M	+++	+++	++±	++±	+++	+++	++±	++±	++±	++±	+++	
Reaction for N	−	−	−	−	−	−	+±	+±	+±	+±	−	

The strength of the reactions is indicated by the signs +, +±, ++, ++±, +++.

TABLE II.

Heredity of the Agglutinogen M.

Type of parents	No. of families	No. of children of type		Per cent of children of type	
		M+	M−	M+	M−
M+ × M+	101	403	33	92.4	7.6
M+ × M−	59	165	85	66.0	34.0
M− × M−	6	0	29	0	100

F = Father; M = Mother.

145					146						147					
M 311	312	313	314	315	F 316	M 317	318	319	320	321	F 322	M 323	324	325	326	327
A	A	A	A	A	O	A	O	A	A	O	A	O	A	A	O	A
++	++	++±	−	++±	++	++±	++±	++±	++±	++±	−	++±	−	++±	−	−
+±	+	+	++	+±	−	++	+±	+±	−	−	++±	++	++±	+±	++±	++

TABLE III.

Heredity of the Agglutinogen N.

Type of parents	No. of families	No. of children of type		Per cent of children of type	
		N+	N−	N+	N−
N+ × N+	31	130	18	88.5	11.5
N+ × N−	29	81	40	66.9	33.1
N− × N−	4	0	17	0	100

The investigations outlined on the heredity of human blood groups are not only of theoretical interest[6] but they have attracted much attention because of their practical application in forensic medicine. A certain limitation lies in the fact that only two properties could be utilized. It is true that some experiments pointed to the existence of differences in human blood aside from the blood groups (13–16), but as a result of these studies no genetic investigations worthy of notice have been reported although obviously such would have been desirable. The reason for this is to be seen in the lack of workable methods.

The observations reported in previous publications (1, 17, 18) enabled us to undertake a study of the heredity of serological properties of human blood other than those determining the four blood groups.

With regard to the property designated as M there was no difficulty in selecting immune sera and absorbing bloods in such a manner that the reactions were either entirely negative on microscopic examination or so strong that clumps were visible to the naked eye.

The results with a second property, N, whose heredity was studied, varied depending upon the particular immune serum used. Although the strongest agglutinations occurred with the same bloods, there were differences in the reactions of minor strength so that the number of positive tests was greater with some sera than with others. In the following experiments two sera were selected which behaved identically and gave the smallest number of positive reactions; *i. e.*, the bloods acted upon by these sera reacted positively with all sera. Moreover with the sera chosen there was a distinct break between positive and negative tests, a point of significance for the present issue.

The frequency of the types M+ and M−, and N+ and N−, as already stated, is approximately the same in the four blood groups. According to our present results, there were in 1708 white individuals 326 (19.1 per cent) with negative reactions for M, and in 532 white individuals there were 139 (26.1 per cent) negative for N.

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that the formula $p + q + r = 1$, holds for arbitrarily chosen values, which is obviously not the case.

[5] While this paper was in press, another explanation based upon the assumption of incomplete linkage was proposed by Bauer (*Klin. Woch.*, 1928, vii, 1588).

[6] *Cf.* Morgan (12).

The technique of performing the tests has been described (18). The absorptions and tests for N were made at about 40°C.

It should be stated that the technique offers some difficulties as compared to the common isoagglutination tests. It is necessary to become well acquainted with the method and to know the properties of each serum in order to absorb completely all agglutinins but those in question.

The material for this study was obtained from two maternity clinics in the City of New York. Altogether 166 families were studied; in most of these (122) there were four or more children. Several families were always included in one experiment and also a number of control bloods with known properties.

TABLE IV.

Unions No.	Type of parents	No. of families	No. of children of types		
			M+N+	M+N−	M−N+
1	M+N+ × M+N+	11	31	17	7
2	M+N+ × M−N+	17	40	1	34
3	M+N+ × M+N−	24	60	40	3
4	M+N− × M−N+	5	17	0	1
5	M+N− × M+N−	4	0	17	0
6	M−N+ × M−N+	3 (6)	0	0	18 (29)

The figures in parentheses in unions of type 6, include the three families tested only for M but which, according to our experience, would be of the type M−N+.

In 166 families only the property M was investigated; 64 families were examined for M and N. A representative experiment is given in Table I.

The results for M are summarized in Table II and are arranged in three classes corresponding to the three types of unions and those for N in Table III are similarly arranged.

64 families were examined both for M and N. The results (Table IV) are arranged according to the six sorts of matings and the three types of offspring that have been observed.

A list of the tests is given in Tables V*a* and V*b* for the individual families, the former showing the tests for M (102 families) and the latter tests for both M and N (64 families). In each case the children are

TABLE V*a*.**

Reactions for M.

Family No.	Father	Mother	Children				
1	O+	B+	O+ ♂	O+ ♂	O+ ♀	O+ ♀	
2	O+	O+	O− ♀	O− ♂	O+ ♂		
3	AB−	AB+	AB− ♀	B+ ♂	B− ♂	A− ♀	
4	A+	A+	A+ ♂ A+ ♂	A+ ♀	A+ ♀	A− ♂	A− ♂
5	A+	A+	A+ ♀	A+ ♂	O+ ♀	A+ ♂	
6	B−	A+	B+ ♀	B+ ♀	B+ ♂	B+ ♂	
7	O+	O+	O+ ♂	O+ ♀	O+ ♂	O+ ♂	
8	AB+	A+	A+ ♂	B+ ♀	A+ ♂	A+ ♂	
9	A+	O−	A+ ♂ O+ ♀	O+ ♀	A+ ♀	O+ ♂	A+ ♀
10	B+	B+	O− ♂	B− ♀	B+ ♂		
11	O+	AB+	A− ♀	A+ ♂			
12	O+	A+	A+ ♀	O+ ♀	A+ ♀	A+ ♂	
13	O−	O−	O− ♀	O− ♀	O− ♀	O− ♀	
14	AB+	A+	A+ ♂	A+ ♂	A+ ♀	AB+ ♂	A+ ♂
15	AB+	A+	A+ ♀	AB+ ♀	AB+ ♂		
16	B+	O−	B− ♀	O− ♂	O+ ♀	O+ ♂	O− ♂
17	A+	O+	A+ ♂	O+ ♀	A+ ♂	O+ ♂	
18	A+	O+	O+ ♀	A+ ♀	A+ ♂*	A+ ♀*	O+ ♀
19	A+	O−	O+ ♀	O+ ♀	A+ ♀	O+ ♀	
20	A−	B+	O+ ♀	AB+ ♀	AB+ ♀	A− ♀	

* Twins.

** 20 of the 166 families examined were negro families.

TABLE V*a*—*Continued.*

Family No.	Father	Mother	Children				
21	A+	O+	A+ ♀	A+ ♀	O+ ♀	O+ ♂	
22	O+	O+	O+ ♀	O+ ♂			
23	O+	O+	O+ ♂	O+ ♂	O+ ♂	O+ ♂	O+ ♂
24	O+	O−	O+ ♂ O+ ♀	O− ♂ O+ ♀	O− ♂	O+ ♀	O+ ♂
25	B+	A−	AB− ♀ B+ ♀	B+ ♀	B+ ♂	AB+ ♀	AB+ ♂
26	AB+	A+	AB− ♂	A+ ♀	AB+ ♂	B+ ♀	B+ ♂
27	O−	A+	O− ♂	A+ ♀	A− ♂	A− ♂	
28	B+	O+	B+ ♂	O+ ♂	B+ ♀	O+ ♂	
29	O+	A−	A+ ♂	O− ♀	O− ♀	A+ ♀	O− ♀
30	O−	O+	O+ ♀	O− ♀			
31	A+	O−	A− ♀	O− ♀	A+ ♀	A+ ♀	
32	A+	O−	A− ♀	A+ ♀	A+ ♂		
33	O−	O+	O− ♂	O+ ♀	O− ♀	O− ♀	
34	B+	A+	A+ ♂	AB+ ♂	AB+ ♀	AB+ ♀	
35	A−	B+	A− ♀	AB− ♂	B− ♂	AB+ ♂	
36	O−	O+	O+ ♂	O+ ♂	O+ ♂	O+ ♂	O+ ♂
37	O+	A+	A+ ♀	A+ ♂	O+ ♀		
38	A+	A+	A+ ♂	O+ ♂	A+ ♂	O+ ♂	
39	O+	A−	A+ ♀	O+ ♂	O+ ♂		
40	O+	A+	A+ ♀	A+ ♀	A+ ♂	A+ ♂	A+ ♀
41	A+	B+	AB+ ♀ AB+ ♂	AB+ ♂	A+ ♀	AB+ ♀	AB+ ♂

TABLE V*a*—*Continued.*

Family No.	Father	Mother	Children				
42	A+	B−	A+ ♂	A− ♀	AB+ ♀	A− ♂	
43	A+	B+	A+ ♀	A+ ♂	AB+ ♀	A+ ♀	
44	B+	A+	A+ ♀	A+ ♂	A+ ♀		
45	B+	O−	O− ♂	B+ ♂			
46	AB−	O+	B+ ♀	B+ ♀*	B+ ♀*	B+ ♀	
47	A+	O+	O+ ♂	A+ ♀	O+ ♂	A+ ♂	O+ ♂
48	A+	A+	O+ ♂	O− ♂	O− ♂		
49	A+	A+	A+ ♀	A+ ♀	O− ♀	A− ♀	
50	B+	A+	B− ♀	O+ ♂	AB+ ♀		
51	O+	O−	O+ ♀	O+ ♀*	O+ ♀*	O+ ♀	
52	O+	O−	O+ ♂	O− ♀	O+ ♂	O− ♂	O− ♂
53	A+	B−	A+ ♀	AB+ ♂	AB− ♂	A− ♂	
54	A+	A+	A+ ♀	A+ ♂	A+ ♀		
55	A+	A+	A+ ♂	A+ ♀			
56	O+	A+	O+ ♂	A+ ♀	O+ ♀		
57	O−	O+	O+ ♀	O+ ♂	O+ ♀	O+ ♂	
58	O+	A+	A+ ♀	A+ ♂	A+ ♀		
59	O+	A+	O+ ♂	O− ♂	O+ ♂	O+ ♀	A+ ♂
60	B−	O−	O− ♂	O− ♂	O− ♀		
61	O+	A−	O+ ♀	O+ ♂	A+ ♀		
62	A+	A+	A+ ♀	O+ ♂	A+ ♂	O+ ♀	
63	O+	A+	O+ ♀	O+ ♂	A+ ♂	A+ ♂	A− ♂

* Twins.

TABLE V*a*—*Continued.*

Family No.	Father	Mother	Children				
64	O+	O+	O+ ♂ O+ ♂	O+ ♂	O+ ♀	O+ ♀	O+ ♂
65	O+	O−	O+ ♂	O+ ♀			
66	O+	B+	B+ ♂	B+ ♂			
67	A+	O+	A+ ♂	O+ ♂			
68	O+	O+	O+ ♀	O− ♂	O+ ♀	O− ♀	
69	A+	O+	O+ ♀	O+ ♀	A+ ♀	O− ♀	A+ ♂
70	A+	O+	O+ ♀ A+ ♂	A+ ♂ O+ ♂	O+ ♀ O+ ♂	O+ ♀ A+ ♀	O+ ♀
71	AB−	O+	O+ ♀ O+ ♀	A+ ♀	B+ ♂	A+ ♂	A+ ♀
72	B+	A−	O+ ♀	O+ ♀	B− ♀	AB+ ♀	
73	A+	O+	O+ ♀	O+ ♀	A− ♂	O+ ♂	
74	O+	A+	A+ ♀	A+ ♂	O+ ♂	O+ ♂	
75	B+	O+	O+ ♂	O+ ♂	B+ ♂	O+ ♀	
76	O+	A+	O+ ♂ O+ ♂	O+ ♂	A+ ♀	A+ ♂	A+ ♀
77	O−	B+	O+ ♂ O+ ♀	O+ ♀	O+ ♂	B+ ♀	O+ ♂
78	O+	O+	O+ ♀ O+ ♀	O+ ♂ O+ ♀	O+ ♂ O+ ♂	O+ ♀	O− ♀
79	O+	B+	B+ ♂	B+ ♂	B+ ♂		
80	O+	B+	O+ ♂	B+ ♀	O+ ♂	B+ ♂	O+ ♂
81	O−	B+	O+ ♀	O+ ♂	B+ ♂		
82	O+	O+	O+ ♂	O+ ♂	O+ ♂		

TABLE V*a*—*Concluded.*

Family No.	Father	Mother	Children				
83	O+	O+	O+ ♂	O− ♀	O+ ♀		
84	O+	O+	O− ♀	O− ♂	O+ ♂	O+ ♂	
85	O+	B−	B− ♂	B+ ♂	B− ♂		
86	A+	B+	B+ ♀	O+ ♀	AB+ ♀		
87	O+	A+	O+ ♀ O+ ♀	A+ ♀	O+ ♂	O− ♀	A+ ♂
88	O+	O−	O+ ♂	O+ ♀	O+ ♂		
89	A+	O−	A+ ♂ O+ ♀	O+ ♂	O+ ♂	A+ ♂	O+ ♂
90	AB−	O+	A− ♂	B− ♀	A+ ♂		
91	O−	AB+	A− ♂ B− ♀	B− ♀	A− ♀	B− ♀	A+ ♀
92	O+	O+	O+ ♀	O+ ♂	O+ ♂	O+ ♂	
93	B+	B+	B+ ♂	B+ ♂	B+ ♂	B+ ♂	
94	O+	A−	A+ ♀	A− ♂	A+ ♂	A− ♂	A+ ♀
95	B+	A+	B+ ♀	B+ ♂	O+ ♂	A+ ♀	
96	A+	A−	O− ♂ O− ♂	A− ♀	A+ ♂	A+ ♀	O− ♂
97	A+	O+	A+ ♂ O+ ♂	A+ ♀	O+ ♀	O+ ♀	O+ ♂
98	O+	A+	O+ ♀	O+ ♀	O+ ♀	O+ ♂	O+ ♀
99	B+	O+	B+ ♂ O+ ♂	B+ ♀	O+ ♀	O+ ♀	O+ ♂
100	A+	A+	A+ ♂	A+ ♂	A+ ♂	A+ ♀	
101	B+	O+	O+ ♂ B+ ♀	B+ ♂ O+ ♀	B+ ♀	O+ ♂	O+ ♂
102	B−	A−	A− ♀	A− ♀	A− ♂	AB− ♀	

TABLE V*b*.

Reactions for M and N.

Family No.	Father	Mother	Children			
103	O++	A++	A++ ♂ A+− ♂	O+− ♀	A−+ ♂	O++ ♂
104	O−+	A++	O−+ ♀	O++ ♂	O++ ♂	A−+ ♂
105	O−+	O−+	O−+ ♀	O−+ ♂	O−+ ♂	O−+ ♂
106	O++	AB+−	B++ ♂	B++ ♀		
107	O+−	A++	O+− ♀	A++ ♂	A+− ♀	A+− ♀
108	O+−	A+−	O+− ♀	A+− ♀	O+− ♂	O+− ♂
109	O+−	A++	A++ ♂ O−+ ♂	O++ ♂	A+− ♂	A+− ♂
110	A++	O−+	A++ ♀	O++ ♀	O++ ♂	O++ ♀
111	A+−	A−+	A++ ♀ A++ ♂	A++ ♂	A++ ♀	A++ ♂
112	O++	O+−	O++ ♂ O++ ♂	O++ ♀	O++ ♀	O+− ♂
113	A−+	A++	A++ ♂	A++ ♀	A−+ ♀	
114	A++	O++	A−+ ♀ O+− ♂	O+− ♂ A++ ♂	O−+ ♀	A++ ♀
115	A+−	A++	A++ ♂	A++ ♀	A++ ♀	A++ ♂
116	O++	B−+	B−+ ♀ O++ ♂	O−+ ♀ B++ ♀	B−+ ♂	O−+ ♂
117	O−+	O++	O++ ♀ O++ ♂	O−+ ♀ O++ ♂	O−+ ♀	O++ ♂
118	O+−	A++	O++ ♀ A+− ♀ A++ ♀	O++ ♂ A++ ♀	O++ ♂ A+− ♀	O+− ♀ O++ ♀
119	O−+	A++	A−+ ♂ A++ ♂	A−+ ♀ A++ ♀	A++ ♀ O++ ♀	O−+ ♀

TABLE V*b*—*Continued.*

Family No.	Father	Mother	Children			
120	A++	O−+	A++ ♂	A++ ♀	A++ ♂	A++ ♀
121	B++	A++	AB−+ ♂ A+− ♂	A++ ♂ A++ ♀	A++ ♀	A++ ♂
122	O+−	A++	O++ ♂ A++ ♂	A+− ♂	A+− ♂	A++ ♀
123	O−+	A+−	A++ ♂	A++ ♀		
124	A+−	A++	A++ ♂ A+− ♀	O+− ♀	A++ ♀	O+− ♂
125	O++	A++	A+− ♂ O++ ♀	A++ ♀	O++ ♀	A++ ♂
126	B++	B++	B++ ♀	B+− ♂	O++ ♂	
127	A+−	B++	B−+ ♂	A+− ♀	O+− ♂	
128	O+−	O++	O+− ♀	O++ ♀	O+− ♀	O++ ♂
129	O++	O+−	O+− ♂ O+− ♀	O+− ♂ O++ ♂	O++ ♀	O++ ♀
130	A−+	A−+	A−+ ♂ A−+ ♂	O−+ ♂ O−+ ♂	A−+ ♀	A−+ ♀
131	A+−	O−+	O++ ♀	O−+ ♀	O++ ♂	O++ ♂
132	A−+	O++	A++ ♀ O++ ♂	O−+ ♀	O−+ ♂	O++ ♂
133	O++	O+−	O+− ♂ O+− ♀	O++ ♀	O+− ♂	O+− ♀
134	O+−	A+−	A+− ♀	A+− ♀	A+− ♂	
135	O+−	O++	O++ ♀	O+− ♂	O++ ♀	
136	A++	A−+	A−+ ♂ O−+ ♀	A−+ ♂	A−+ ♀	A−+ ♀
137	B+−	O++	B++ ♂	B+− ♀		

TABLE V*b*—*Continued.*

Family No.	Father	Mother	Children			
138	A+−	O++	O++ ♀	A+− ♀		
139	A+−	B−+	B++ ♀	B++ ♀	B++ ♀	AB++ ♀
140	A++	B++	O++ ♀ O++ ♀	O++ ♂ O++ ♂	A++ ♂	AB+− ♂
141	O++	B++	B++ ♀ O++ ♂	B++ ♂	O++ ♀	O−+ ♂
142	O+−	O++	O++ ♂ O−+ ♂	O++ ♂	O++ ♀	O++ ♂
143	A+−	A+−	A+− ♂	A+− ♀	A+− ♂	A+− ♂
144	O++	O++	O++ ♀	O++ ♀	O+− ♀	O++ ♂
145	O−+	A++	A++ ♂	A++ ♂	A−+ ♀	A++ ♀
146	O+−	A++	O++ ♀	A++ ♂	A+− ♂	O+− ♂
147	A−+	O++	A−+ ♀	A++ ♂	O−+ ♀	A−+ ♂
148	A++	B−+	B−+ ♀	A++ ♂		
149	O−+	B−+	B−+ ♂ O−+ ♂	O−+ ♀ B−+ ♂	O−+ ♀ O−+ ♂	B−+ ♂ B−+ ♀
150	O−+	A++	A−+ ♀	A++ ♀	A+− ♂	A−+ ♀
151	B+−	O−+	O++ ♀	B++ ♀	B++ ♀	
152	O−+	O++	O−+ ♂	O++ ♂		
153	A−+	O++	A++ ♂ O++ ♀	A−+ ♀ O−+ ♀	O−+ ♂	O−+ ♂
154	A++	B+−	O++ ♀	B++ ♀	O+− ♂	
155	A+−	A++	A++ ♂ A+− ♂	A+− ♂ A++ ♂	A++ ♂	A+− ♂
156	A++	O++	O++ ♀	O+− ♀	O+− ♂	A+− ♀

TABLE V*b*—*Concluded.*

Family No.	Father	Mother	Children			
157	O−+	O++	O++ ♀ O++ ♂ O−+ ♀ O−+ ♀ O++ ♀ O−+ ♂			
158	O++	A−+	A++ ♀	A++ ♂	O++ ♂	
159	O++	B+−	B++ ♀	O++ ♀	B++ ♀	B++ ♂
160	A++	O++	A+− ♂	A++ ♀	O+− ♂	A−+ ♂ O++ ♂ O+− ♀
161	O++	A++	A++ ♀	A++ ♀	A+− ♀	A+− ♂ A−+ ♀
162	A+−	O++	A++ ♂	A++ ♂	A+− ♀	A+− ♂ O++ ♀
163	B+−	O+−	O+− ♂	B+− ♂	O+− ♂	B+− ♂ O+− ♂ B+− ♂
164	B+−	A++	O++ ♀	A++ ♂	A++ ♂	O++ ♂ AB++ ♂
165	B+−	O++	O++ ♀	O+− ♀	B+− ♀	O+− ♀
166	B+−	O++	B++ ♀	O++ ♀	B+− ♂	

recorded in order of decreasing age beginning with the eldest. The letters designate the groups, and the signs + and − the reactions for M (Table V*a*). In Table V*b* the first + or − sign designates the test for M and the second sign that for N.

As to the heredity of the factors A and B our results agree with the established fact that they are inherited as Mendelian dominants, except for three families in which A or B appeared in children when they were absent in the blood of the parents. These cases were considered as instances of illegitimacy and were excluded from the tabulations. One of these families was examined only for M and two for both M and N. The results were:

Father	Mother	Children			
O+	O+	O+♂	B+♂	O+♀	A+♀
O++	O++	A++♀	A++♀	O++♀	O+−♀
O+−	B−+	AB++♀	O++♂	O++♀	O++♀

In family 71, AB × O, there were two children in group O; the mother refused reexamination.

From the data reported it is evident that the agglutinogens studied are inherited properties. If we consider M and N separately they would seem to behave like Mendelian dominants. The characters cannot be recessive since in unions + × + there are children whose blood lacks the property. This result is to be expected if there are individuals among the parents heterozygous for a dominant character. If the absence of the agglutinogens is recessive there should occur no positive reactions in children from unions − × −. This is actually borne out by the observations. Thus in six such families with M− parents all the children (29 in number) gave negative reactions for M; likewise in the four families with N− parents all the children (17 in number) belonged to the N− type. In this respect our findings are analogous to the rule established by von Dungern and Hirschfeld for the isoagglutinogens A and B, *i. e.*, that these do not appear in the offspring if they are absent in both parents.

In order to discuss the numerical results for M alone in the three sorts of matings it is necessary to know the incidence of homozygous (MM) and heterozygous (Mm) individuals among the M+ parents. From a formula quoted by Johannsen (19), the following values are obtained: MM = 29.6; Mm = 49.6; mm = 20.8 (approximately 30, 50, 20, respectively). According to this formula the percentage of homozygous individuals equals $100 - 20\sqrt{R} + R$, that of heterozygous $20\sqrt{R} - 2R$; where R is the percentage of recessive (M−) individuals observed in the population. The figures of M+ and M− reactions are taken from all individuals, including the parents, of the 166 families examined for M.

Calculating from these figures the number of M− children in the unions M+ × M+ one has to consider only those in which both parents are heterozygous, *i. e.*, approximately 5/8 × 5/8; since 1/4 of the children of these matings should be M−, 9.8 per cent of M− children are to be expected; the observed value is 7.6 per cent.

In the unions M+ × M− 50 per cent of the offspring of heterozygous M+ parents may be expected to be M−; *i. e.*, 5/8 × 1/2 = 31.3 per cent; actually 34 per cent M− children were found.

In the smaller series where both factors were examined (see Table IV) the agreement between the calculated and the observed values is not so satisfactory.

Applying the formula of Johannsen for the factor N we obtain NN = 23.1; Nn = 49.8; nn = 27.1. Calculated as above the figures are in matings N+ × N+ 11.7 per cent N− children (observed 11.5 per cent) and in matings N+ × N− 34.2 per cent N− children (observed 33.1 per cent).

So far the cursory analysis of the results does not contradict the idea of two independent factors. However, there is evidence which does not seem compatible with this view. In the first place, if the factors were independent one would expect a certain, although small, percentage of bloods to lack both M and N, that is, if the genotype M−N− is not lethal, or its phenotype indistinguishable from one of the other types. In fact, as has been stated formerly (18) no such case has been found in the examination of more than 1200 specimens[7] and in each M− blood the reaction for N was found to be very strong. Further evidence emerges from an analysis of results in families examined for both properties. One sees that the frequency of M− children in the three sorts of matings, Nos. 1, 3, and 5 (Table IV) of parents M+, varies greatly according to the N reactions of the parents and that likewise the occurrence of children N− in matings 1, 2, and 6 is influenced by the presence or absence of M in the parents. A similar statement holds for the appearance of M− or N− children in matings 2 and 4, and 3 and 4, respectively.

Actually in most of the six sorts of unions the observed figures do not tally satisfactorily with those to be expected on the basis of two independent factors if one computes the expectancy from the figures given above for heterozygous and homozygous individuals. Thus in matings 2 and 3 there are too many children of the type M−N+ or M+N−, respectively, and in union 4 there appear with one exception only children of type M+N+. These numerical results could be interpreted in various ways. One hypothesis consists in assuming two genes which, when homozygous, would determine the phenotype M+N− and M−N+ respectively, while the phenotype M+N+

[7] This number includes the blood of negroes and Indians. About 900 of these were tested with the improved technique, namely, performing the tests for N at about 37–40°C.

would correspond to the heterozygous gene constitution. This view accounts for the non-existence of the type M−N−. On the basis of this hypothesis the expected values for the types of offspring are: mating 1: M+N+ 50 per cent, M+N− 25 per cent, M−N+ 25 per cent; mating 2: M+N+ 50 per cent, M−N+ 50 per cent; mating 3: M+N+ 50 per cent, M+N− 50 per cent; mating 4: M+N+ 100 per cent; mating 5: M+N− 100 per cent; mating 6: M−N+ 100 per cent.[8] Allowing for the relatively small number of individuals examined these figures agree fairly with those observed and better than the figures calculated for independent factors. Especially striking is the fact that in matings 2 and 3 there are almost no children M+N− or M−N+, respectively, and in mating 4 only one child not of the type M+N+. Still, there are five cases which contradict the hypothesis mentioned, namely, the individual M+N− in union 2, the three children M−N+ in union 3, and one child M−N+ in union 4. To attribute all these five exceptions to illegitimacy seems hazardous since only a proportion of illegitimate children would be detected by the tests employed and because the number of the exceptional cases is high compared with that of children which do not conform to the rule of von Dungern and Hirschfeld.

It may be pointed out that in all of the five exceptional cases in unions 2, 3, and 4, the father and not the mother is of the type opposite to that of the child, *e. g.*, father +−, child −+.

On the basis of the assumption just discussed and with the aid of the formula used above, the frequency of one type could be used to calculate the frequency of the other two types. Starting from the figure 20.8 for M− in a certain population the computation gave the value of 29.6 N−; similarly in a population of 205 Indians examined by us, the observed value of 4.9 M− leads to an expectancy of 60.7 for N−. Both these figures are in good agreement with those observed, namely, 26.1 and 60.0 respectively.[9]

An alternative hypothesis would suppose a close linkage between M and N. If, then, the gene combinations Mn and mN are numerically predominant this could explain the observed figures and also the occurrence of the exceptional cases aforementioned, but unless one assumes a lethal effect there arises a difficulty from the following con-

[8] The numbers of the matings refer to those given in Table IV.

[9] These results will be discussed in another publication (20).

sideration. If the factors M and N have been in the race for a long time the occurrence of cross-overs should by now have reduced the assumed numerical difference. However, on account of the existence of agglutinogens similar to M and N in anthropoids (chimpanzees) (18) it does not seem likely that they are due to recent mutations.

If in the unions listed as No. 1, Table IV, one of the parents be homozygous with respect to both M and N, then all children of such a union would be of the phenotype M+N+. The fact that this was not the case in any of the eleven families shows that none of the parents can be homozygous for both M and N, but such homozygous parents may have occurred in unions 2 and 3.

In view of the limited number of families studied it would seem premature to attempt a final interpretation and to discuss further possibilities such as the existence of more than two allelomorphs. Also it has to be considered that the state of affairs may be complicated, *e. g.*, by interacting or modifying effects of factors determining hitherto unknown agglutinable structures.

It may be added that there is no indication of a linkage between M and N and the isoagglutinogens A and B, and, as in the case of A and B, no evidence of a sex linkage.

SUMMARY.

The heredity of two agglutinable structures demonstrable by immune agglutinins was studied in 166 families. From the data collected it is evident that one deals with a case of Mendelian inheritance. The main result of the studies is the demonstration that it is feasible to investigate the heredity of serological structures of human blood other than the group agglutinogens. Irrespective of the ultimate theory it seems very probable that the properties M and N do not appear in the offspring when they are absent in both parents—a conclusion substantiated by the examination of ten families with 46 children. These findings offer the prospect of forensic application to cases of disputed paternity and, in our opinion, a correct decision could already be given, at least with great probability, provided the reagents are available and the method properly applied. Of course further work is needed before the test can be adopted as a routine procedure.

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